The role of PKN in cell movement

Amy Louise Jevons

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University College
University of London

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London Research Institute
Cancer Research UK
44 Lincoln's Inn Fields
London
WC2A 3PX

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Declaration: I, Amy Louise Jevons, confirm that the work presented in this thesis is my own. Where information has been derived from other sources this has been indicated in the text.

Abstract

This thesis focuses on the protein kinase N family of serine/threonine protein kinases. This comprises three isoforms, with kinase domains highly related to the protein kinase C family, but with distinctive Rho/Rac dependent regulation. The critical roles played by Rho/Rac in regulating the cytoskeleton and cell migration/invasion led to an investigation into the role of PKNs in this process.

Initial work in the thesis derived from the observation that PKN1 translocated to an insoluble cytosolic compartment in response to hyperosmotic stress. This had been shown to be dependent upon Rac1 and 3-phosphoinositide dependent kinase (PDK1). The thesis describes the characterisation of the domain in PKN1 responsible for the Rac-dependent hyperosmotic response. Through the use of PKN1/PKCzeta chimeras a 49 amino acid sequence within the kinase domain was shown to be necessary and sufficient for the observed osmotic behaviour.

In developing a cell model for migration/invasion, a breast cancer cell model was identified that displayed high PKN expression. siRNA mediated depletion of PKN isoforms or inhibition of kinase activity, revealed a requirement for PKN in both migration and invasion. Comparison with other transformed cells indicated that the relative contribution each isoform makes towards these processes appears to be cell type dependent.

As a candidate downstream target, PLD has been implicated in migration and invasion of breast cancer cells and so the previously described interaction between PKN and phospholipaseD in migrating breast cancer cells was investigated. Results suggest that the activity of PLD contributes to the migration of MDAMB-468 cells and that the production of phosphatidic acid in migrating cells is stimulated by PKN.

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Finally I'd like to dedicate this thesis to my Mum, Dad, Dan and Lena even though I doubt they'll ever read it! I love them all very much.

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Abbreviations

ATP	adenosine triphosphate
BSA	bovine serum albumin
BtOH	butanol
Bt-2-OH	isobutanol
cAMP	cyclic AMP
Ca^{2+}	calcium ions
CO	collagen
DAG	diacyclglycerol
DNA	deoxyribonucleic acid
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EGF(R)	epidermal growth factor (receptor)
EMT	epithelial-mesenchymal transition
EtOH	ethanol
FAK	focal adhesion kinase
FCS	foetal calf serum
FN	fibronectin
GAPs	GTPase activating proteins
GDIs	guanine nucleotide dissociation inhibitors
GDP	guanosine 5'-diphosphate
GEFs	guanine nucleotide exchange factors
GFP	green fluorescent protein
GTP	guanosine 5'-triphosphate
GTPase	GTP hydrolase
Hrs	hours
IP_3	inositol 1,4,5-triphosphate
kDa	kilo Daltons
M	molar
m	mili

mins	minutes
μ	micro
MAPK	mitogen activated protein kinase
MEFs	mouse embryonic fibroblasts
Mins	minutes
MLC	myosin light chain
MMPs	matrix metalloproteinases
LC-MS/MS	liquid chromatography tandem mass spectrometry
LN	laminin
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDK1	phosphoinositide dependent kinase
PH	pleckstrin homology domain
PLC/D	phospholipaseC/D
PI	phosphoinositide
PI3-K	phosphatidylinositol 3-kinase
PKB	Protein kinase B
PKC	Protein kinase C
PtdCho	phosphatidylcholine
PtdEt	phosphatidylethanolamine
PtdIns	phosphatidylinositol
PtdIns3P	phosphatidylinositol 3 phosphate
PtdIns(3,4)P_2	phosphatidylinositol (3,4) bisphosphate
PtdIns(3,4,5)P_3	phosphatidylinositol (3,4,5) triphosphate
PKN	Protein kinase N
PtdOH	phosphatidic acid
PtdSer	phosphatidylserine
PTEN	phosphatase and tensin homolog deleted on chromosome ten
PTMs	post-translational modifications
ROCK	Rho kinase

RT	room temperature
rpm	revolutions per minute
SH2/3	Src homology 2/3 domain
Ser	serine
Thr	threonine
Tyr	tyrosine
VEGF(R)	vascular endothelial growth factor (receptor)
v/v	volume/volume
WB	western blotting
w/v	weight/volume
x	times

Chapter 1

Signal transduction

1 Introduction

All cells must regulate their behaviour in response to cues from their environment. This conversion of a specific signal or stimulus into a relevant response is termed signal transduction and is essential for the correct functioning and survival of both single-celled and multicellular organisms. Receptors detect these signals and as a result of their activation the activity and/or localisation of multiple intracellular mediators are affected. These linked series of events are termed signalling pathways and the interplay between different pathways generates complex signalling networks that ultimately result in an appropriate biological response to the signal. Since it is these signalling networks that control the functioning of all tissues and organs of a multicellular organism it is not surprising that defects in cell signalling often results in disease. The elucidation of these signalling pathways is therefore critical to allow therapeutics to be developed to treat such diseases. Some of the general aspects of signal transduction (illustrated in Figure 1:1) will be described here with particular emphasis on areas relevant to this thesis.

Figure 1:1 A generic signal transduction pathway (Downward, 2001).

Schematic illustrating some of the important concepts of signal transduction. The grey boxes indicate general components of signalling pathways, the white boxes show specific examples.

1.1 Receptors

The signals that are received by cells arrive in the form of ligands that bind to specific receptors. The binding of a ligand to its receptor results in changes to the conformation of the receptor that result in transduction and amplification of the signal. The majority of ligands are too large and/or too polar to be able to pass through the membrane lipid bilayer and so cell surface receptors provide the means for the signal to be transduced across the membrane. Other ligands, such as the steroid hormones, are able to pass through the membrane and bind to intracellular receptors that then frequently enter the nucleus and bind to DNA to bring about changes in gene expression (see (Tata, 2002) for review). Receptors that mediate cell-cell and cell-matrix adhesion are of particular interest to this thesis and are discussed in more detail below.

1.1.1Cadherins

The cadherins are a group of Ca^{2+} dependent transmembrane receptors that usually mediate homotypic cell-cell adhesion by acting as both ligand and receptor to form cell contacts. There are many classes of cadherins and they are named according to the tissue type that they are associated with e.g. E-cadherin is primarily found in cells of epithelial origin. Their ability to mediate adhesion between cells that are expressing the same subclass of cadherin means that they play a vital role in tissue organisation in development and maintaining the integrity of multicellular organs (for review see (Ivanov et al., 2001)). At points of cell contact cadherins are dynamically linked to the cytoskeleton and intracellular signalling events through proteins such as the catenins binding to their cytoplasmic tails (Mege et al., 2006). The regulation of cell contacts is the subject of much investigation not least because loss of cell-cell contacts and the ability of cells to migrate as single cells rather than in a collective fashion is a hallmark of the epithelial-mesenchymal transition (EMT, (Boyer et al., 2000) for review). This process has been associated with tumour progression and interestingly loss of E-cadherin expression or regulation has been reported in many aggressive tumours (Foty and Steinberg, 2004).

1.1.2 Integrins

Integrins are a large family of heterodimeric receptors that consist of an α and a β subunit. Both subunits contain a large extracellular domain, a membrane spanning region and a smaller intracellular domain. The extracellular domains associate noncovalently to form a functional receptor. There are currently 18 known α subunits and 8 β subunits and these combine in various combinations to give 24 integrin heterodimers (for recent review see (Humphries et al., 2006)). The integrins have overlapping ligand binding specificities and many integrins can bind multiple extracellular ligands. The classical integrin binding motif found in ligands such as fibronectin and vitronectin is the arginine-glycine-aspartic acid (RGD) sequence. However most integrins do not recognise this sequence and indeed many integrins that bind fibronectin do so through other sites. Frequently though the recognition sites are short linear sequences containing an acidic residue (Ivaska and Heino, 2000). Table 1 summarises the main ligand(s) for each integrin.

Multiple proteins are able to bind to the cytoplasmic tails of integrins and this creates focal adhesion sites. Binding of scaffolding proteins such as paxillin, α-actinin and vinculin provide a link between the extracellular matrix and the cytoskeleton. Integrins lack intrinsic catalytic activity and so recruitment of proteins such as GTPases, kinases and phosphatases to these focal adhesion sites provide a link between the ECM, cytoskeleton and intracellular signalling. This enables integrins to control aspects of cell behaviour such as migration, invasion, proliferation and survival (reviewed by (Geiger et al., 2001)). The importance of the dynamic regulation of these focal adhesion sites for cell migration will be discussed later.

INTEGRIN	LIGANDS
α1β1	LN, CO
α2β1	LN, CO, thrombospondin, E-cadherin, tenascin
α3β1	LN, CO, thrombospondin, uPAR
α4β1	Thrombospondin, MAdCAM-1, VCAM-1, FN, osteopontin, ADAM, ICAM-4.
α5β1	FN, osteopontin, fibrillin, thrombospondin, ADAM, COMP, L1
α6β1	LN, thrombospondin, ADAM, Cyr61
α7β1	LN
α8β1	Tenascin, FN, osteopontin, VN, LAP-TGF-β, nephronectin
α9β1	Tenascin, VCAM-1, osteopontin, uPAR,plasmin, angiostatin, ADAM, VEGF-C,VEGF-D
α10β1	LN, CO
α11β1	LN
αVβ1	LAP-TGF-β, FN, osteopontin, L1, VN
αLβ2	ICAM, ICAM-4
αMβ2	ICAM, iC3b, factor X, fibrinogen, ICAM-4, heparin
αXβ2	ICAM, iC3b, fibrinogen, ICAM-4, heparin, CO
αDβ2	ICAM, VCAM-1, fibrinogen, FN, VN, Cyr61, plasminogen
αIIbβ3	Fibrinogen, thrombospondin, FN, VN, vWF, Cyr61, ICAM-4, L1, CD40 ligand
αVβ3	Fibrinogen, VN, vWF, thrombospondin, fibrillin, tenascin, PECAM-1, FN, osteopontin,

	BSP, MFG-E8, ADAM-15, COMP, Cyr61, ICAM-4, MMP, FGF-2, uPA, uPAR, L1, angiostatin, plasmin, cardiotoxin, LAP-TGF-β, Del-1
α6β4	LN
αVβ5	Osteopontin, BSP, VN, CCN3, LAP-TGF-β
αVβ6	LAP-TGF-β, FN, osteopontin, ADAM
α4β7	MAdCAM-1, VCAM-1, FN, osteopontin
αEβ7	E-cadherin
αVβ8	LAP-TGF-β, FN, VN

Table 1 Known ligands for the 24 identified integrin dimers.

Reviewed in (Takada et al., 2007) (Ivaska and Heino, 2000).
Abbreviations: FN, fibronectin; LN, laminin; CO, collagen; VN, vitronectin; ICAM, intercellular adhesion molecule; VCAM, vascular cell adhesion molecule; iC3b, inactivated C3b; MadCAM-1, mucosal addressin cell adhesion molecule-1; ADAM, a disintegrin and metalloprotease; BSP, bone sialic protein; CCN3, an extracellular matrix protein; COMP, cartilage oligomeric matrix protein; Cyr61, cysteine rich protein 61; L1, CD171; LAP-TGF-β, TGF-β latency-associated peptide; PECAM-1, platelet and endothelial cell adhesion molecule 1; uPA, urokinase; uPAR, urokinase receptor; VEGF, vascular endothelial growth factor; vWF, von Willebrand Factor.

1.2 Second messengers

Second messengers are molecules that relay and amplify the signals received by receptors to generate rapid and diverse responses. The activation of a single receptor will often result in the generation of multiple second messenger molecules and hence the signal is amplified (Eyster, 1998). These second messengers are frequently able to diffuse to other locations within a cell and so influence signalling distal to the location of the receptor. In addition second messengers can affect multiple proteins in the cell and therefore multiple signalling pathways can be activated in response to a single signal allowing integration and fine-tuning of responses. Prevalent second messengers include cyclic AMP (cAMP) (Lynch et al., 2006), diacyclglycerol (DAG) (Carrasco and Merida, 2007), calcium (Ca^{2+}) and inositol 1,4,5-triphosphate (IP_3) (Brini and Carafoli, 2000). The effect of receptor activation and second messenger generation is to affect the activity and interaction of proteins to generate signalling cascades. The mechanisms by which these changes are brought about are discussed below.

1.3 Post-translational modifications

Many polypeptide chains are chemically modified after they are translated to alter the function of the resulting protein. These modifications can involve the addition of biochemical groups, addition of polypeptides, altering the chemical nature of amino acids or structural changes to the protein (Zhu et al., 2003). Together these modifications add to the diversity of functions that a protein can have. They also enable greater control over the response to a particular signal to be achieved by regulating the location and activity of the proteins within a signalling pathway. Although there are over 200 post-translational modifications (PTMs) one of the most important and studied is that of protein phosphorylation.

1.3.1 Protein Phosphorylation

Protein phosphorylation is the addition of a phosphate group to specific residues of a protein. The transfer of the γ-phosphate group from ATP onto the hydoxyl group of specific residues within a protein, most commonly a serine, threonine or tyrosine residue, is catalysed by protein kinases. The addition of a single phosphate can dramatically change the activity of a protein although many proteins are phosphorylated on multiple residues. The beauty of phosphorylation as a molecular "on/off" switch for signalling pathways arises due to the opposing actions of kinases and phosphatases, (that catalyse the removal of phosphates from proteins), allowing exquisite control of the activity and interactions of their target proteins. The completion of the human genome project has revealed that there are more than 500 kinases and over 100 phosphatases illustrating their importance in controlling cellular activity (Mann et al., 2002).

Kinases can be grouped into three main classes based on the residues they phosphorylate, the serine/threonine (Ser/Thr) kinases, tyrosine (Tyr) kinases and the dual specificity kinases. They have been further divided into families based on sequence comparisons of their catalytic domains, sequence and domain structure outside of the catalytic domain and biological function (Manning et al., 2002).

All the classical kinases have a common bilobal catalytic domain of approximately 250 amino acids. The small N-terminal region consists mainly of β-sheets and the larger C-terminal lobe contains primarily α-helices. ATP binds in a cleft between the two lobes that the substrate then binds along (Hanks and Hunter, 1995). Conserved residues in the kinase domain then catalyse the transfer of the γ-phosphate to the appropriate residue of the substrate. Three motifs within the catalytic domain of a kinase are thought to be critical for activity (Table 2); 48 proteins have been identified so far that lack one or more of these motifs and these pseudokinases are thought to be catalytically inactive (Boudeau et al., 2006).

Motif	Function
VAI**K** (Val-Ala-Ile-Lys)	Lysine residue interacts with α and β phosphates of ATP to anchor and orientate the ATP.
HR**D** (His-Arg-Asp)	Aspartic acid residue functions as a base receptor to achieve proton transfer.
DFG (Asp-Phe-Gly)	Aspartic acid residue binds the Mg^{2+} ions that coordinate the β and γ phosphates of ATP in the binding cleft.

Table 2 Critical kinase domain motifs.

It is estimated that at any time a third of cellular proteins are phosphorylated with a phosphoamino acid content ratio (pSer:pThr:pTyr) of 1800:200:1 (Mann et al., 2002). Phosphorylation can affect proteins in several ways to result in regulation of signal transduction pathways. The addition of a bulky negatively charged phosphate group commonly alters the conformation and hence the activity of a protein. For example members of the Protein kinase C (PKC) family are activated by phosphorylation on their activation loop residue (see section 1.8) (Parker and Parkinson, 2001), the Tyr kinase Src is regulated both positively and negatively by phosphorylation of multiple residues (Roskoski, 2005). Altering the activity of a single enzyme such as a kinase can have pronounced effects on signalling by affecting the phosphorylation of multiple downstream targets of that enzyme producing a signalling "cascade". In addition to affecting activity, phosphorylation can also control the localisation of proteins. For example the Forkhead boxO (FoxO) transcription factors when phosphorylated by PKB are sequestered to the cytoplasm where they are unable to regulate gene expression (Kops and Burgering, 1999). Phosphorylation can also creating docking sites for protein binding domains on other cellular proteins and hence promote protein-protein interactions to regulate the propagation of a signal, this is discussed in section 1.5.

The balance of phosphorylation is achieved through the kinase opposing action of phosphatases. Like the kinases there are three classes of phosphatases, Ser/Thr phosphatases, Tyr phosphatases and dual specificity phosphatases. The Ser/Thr phosphatases are vastly outnumbered by the Ser/Thr kinases. Therefore to achieve specificity and regulation of Ser/Thr residue dephosphorylation multiple regulatory subunits combine with core catalytic subunits to create distinct holoenzymes with a wide variety of substrate specificities and subcellular localisations (Barford et al., 1998). The tyrosine phosphatases however do not associate with regulatory subunits but achieve specificity of function through the presence of multiple regulatory and interaction domains (Alonso et al., 2004).

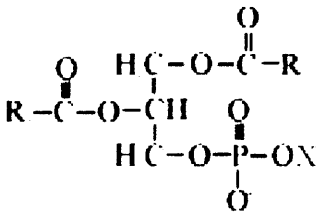
To achieve the correct response to a specific signal kinases and phosphatases must distinguish which residues to phosphorylate and dephosphorylate respectively from amongst thousands of possibilities within the cell. This specificity of action is achieved through multiple mechanisms such as structure of the catalytic site, local interactions with the substrate and the binding of scaffolding and adaptor proteins ((Ubersax and Ferrell, 2007) for review). The localisation of kinases and the interactions with protein and lipid partners to generate signalling complexes is particularly relevant to the work in this thesis.

1.4 Phospholipids

Phospholipids are the main constituent of membrane bilayers and as such are vitally important in insulating cells from the external environment and creating cellular compartments with different characteristics to the cytoplasm. However they also play an important role in signalling both by targeting proteins to specific cellular compartments and generating second messengers (recently reviewed (Eyster, 2007)).
The phospholipids consist primarily of the glycerophospholipids. These all have two fatty acid chains attached to the first and second carbons of a glycerol molecule and a

phosphate group attached to the third carbon (this is the parent compound, phosphatidic acid). The identity of the head group that is attached to this phosphate subdivides the glycerophospholipids into phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine, phosphatidylglycerol and phosphatidylinositol (see Figure 1:2). The fatty acids in each type of glycerophospholipid and the nature of the fatty acid linkage can vary and so give rise to a number of molecular species. The composition of species found varies between organisms and between and within cells of the same organism. The phosphoinositides (PIs) are particularly important in signalling and their role will now be considered in more detail.

Basic phospholipid structure



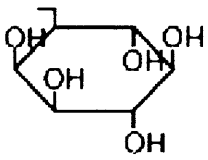
Formula of X	Name of phospholipid
$-CH_2CH_2NH_3^+$	**Phosphatidylethanolamine**
$-CH_2CH_2N(CH_3)_3^+$	**Phosphatidylcholine**
$-CH_2CH(OH)CH_2OH$	**Phosphatidylglycerol**
	**Phosphatidylinositol**

Figure 1:2 Illustration of the different types of phospholipid.

1.4.1 Phosphoinositides

The differential phosphorylation of the inositol ring at positions 3, 4 or 5 generates seven different phosphatidyl inositol (PtdIns) lipids in higher eukaryotes. As well as giving rise to a number of second messengers, such as IP3, DAG and PtdOH, the PIs also serve to recruit proteins containing lipid interaction domains to specific membrane compartments or areas of a membrane (Krauss and Haucke, 2007). Specific PI kinases and phosphatases act to interconvert the phosphoinositides (PIs) and hence regulate their function. The kinases are named after the position on the inositol ring that they phosphorylate i.e. PI3, PI4 and PI5 kinases. The PI3-kinase signalling pathway is the best understood and has been implicated in a huge range of cellular processes such as cell growth, proliferation, survival and cytoskeletal changes (for review see (Vanhaesebroeck et al., 2001)).

Eight mammalian PI3-kinases have been identified and they are grouped into three classes of PI3 kinases based on structural homology, regulation and substrate specificity (Anderson and Jackson, 2003). In conjunction with other lipid kinases they can generate three lipid products, PtdIns3P, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3. The class I PI3-kinases are the best characterised and can produce all three phosphorylated phosphoinositides but are thought to preferentially phosphorylate PtdIns(4,5)P_2 *in vivo* to give rise to PtdIns(3,4,5)P_3. The class 1a group are heterodimeric proteins consisting of a 110kDa catalytic subunit and a adaptor subunit (usually 85kDa). The regulatory subunit whilst having no intrinsic catalytic activity contains multiple protein interaction domains such as SH3, SH2 and proline rich regions (see section 1.5) that are essential for the correct functioning of the PI3-kinases. The class 1b enzyme comprises a catalytic and distinct regulatory subunit (p101 or p87) that confers G-protein sensitivity. Class I PI3-kinases are primarily activated downstream of Tyr kinase receptors in response to growth factor stimulation but they are also activated downstream of integrins, G-protein coupled receptors and intracellular Tyr kinases (for review see (Anderson and Jackson, 2003)). Activation of the kinase is achieved through binding to specific phosphotyrosine residues mediated through the p85 SH2 domains. Binding to these residues brings the kinase into close proximity of its substrate PtdIns(4,5)P_2 in cellular membranes and

results in the production of PtdIns(3,4,5)P_3. Signalling events downstream of PI3-kinase are regulated through the recruitment to specific areas of the membrane of proteins such as phosphoinositide-dependent kinase 1 (PDK1) and protein kinase B (PKB). These proteins contain PH domains that bind to PtdIns(3,4,5)P_3 (Cantrell, 2001). Once at the membrane PDK1 is activated and can then phosphorylate multiple downstream targets such as PKB that are also recruited to the membrane through lipid interaction domains such as PH, PX and FYVE domains. PI3-kinase signalling therefore affects a wide range of cellular activities. In particular PI3-kinase activation is the result of growth factor receptor signalling and promotes cell proliferation. However cell growth must be tightly regulated and the action of PI3-kinase is opposed by the phosphatase PTEN (phosphatase and tensin homolog deleted on chromosome ten). PTEN dephosphorylates PtdIns(3,4,5)P_3 at the 3 position of the inositol ring and so down-regulates PI3-kinase mediated signalling pathways. Disruption of the PI3-kinase pathway, either by inactivation of PTEN or hyperactivation of PI3-kinase, can lead to a cell displaying all of the six hallmarks of cancer (see section 1.7.1) (Dillon et al., 2007) demonstrating the important and diverse roles the pathway plays in controlling cell behaviour.

1.4.2 Phospholipase D

Phospholipases catalyse the hydrolysis of ester bonds in phospholipids and are grouped into four classes based on the specific bond that they hydrolyse. They participate in signal transduction by giving rise to second messenger molecules (see 1.2). For example, activation of receptor tyrosine kinases by growth factors often results in the recruitment and activation of Phospholipase C (PLC) at the plasma membrane where it can hydrolyse its substrate PtdIns(4,5) P_2 to produce the second messengers DAG and IP_3 (Eyster, 2007). The other eukaryotic classes of phospholipase are PLA1, PLA2 and PLD, PLD has particular relevance to the work presented in this thesis (for review see (Liscovitch et al., 2000)).

So far two mammalian PLD genes have been identified, PLD1 and PLD2, each of which has two splice variants. The substrate for PLD is phosphatidylcholine (PtdCho) and the

action of PLD results in the formation of PtdOH and choline. PtdOH functions as a second messenger and can also be converted to DAG, another important second messenger, through the action of phosphatidate phosphohydrolase. Little is known about cell type expression patterns or the substrate specificities for the different isoforms but presumably they have evolved to have different signalling roles (for review (Meier et al., 1999)). Both isoforms have a PX and a PH domain (see 1.5) that bind lipids such as PtdIns(4,5)P_2 to both activate the enzymes and target them to specific membranes. A range of agonists have been reported to activate PLD such as insulin (Huang et al., 2005), PDGF and EGF (Sung et al., 2001). The PLD isoforms particularly PLD1 have been shown to be activated by small GTPases such as RhoA, Cdc42 and Arf (Henage et al., 2006) (Yoon et al., 2006) (Meacci et al., 1999). PLD1 has also been shown to be activated by PKCα (Henage et al., 2006) and PKN1(Oishi et al., 2001).

PLD signalling has been implicated in cellular processes such as membrane trafficking events (Lee et al., 2006a) (Corrotte et al., 2006), reorganisation of the actin cytoskeleton (Cross et al., 1996; Ha and Exton, 1993) and shown to have an anti-apoptosis role (Oh et al., 2007) (Lee et al., 2006b). Of particular interest to the work presented here PLD isoforms have also been shown to play a role in controlling migration. For example in MDAMB-231 cells PLD activity increased upon serum withdrawal and this also promoted migration and invasion (Zheng et al., 2006). PLD2 was shown to be required for LPA induced migration of fibroblasts (Pilquil et al., 2006) and both PLD1 and PLD2 were shown to contribute to the migration of leukocytes (Lehman et al., 2006).

The ability of the PLD isoforms to be involved in multiple signalling processes is achieved through regulation of their activity both temporally and spatially. The presence of lipid interaction domains means that they can be targeted to specific areas of cell membranes where other components of the activated pathway are also found and so the signal can be propagated. Protein interaction domains aid the formation of signalling complexes at specific cellular locations and these are discussed below.

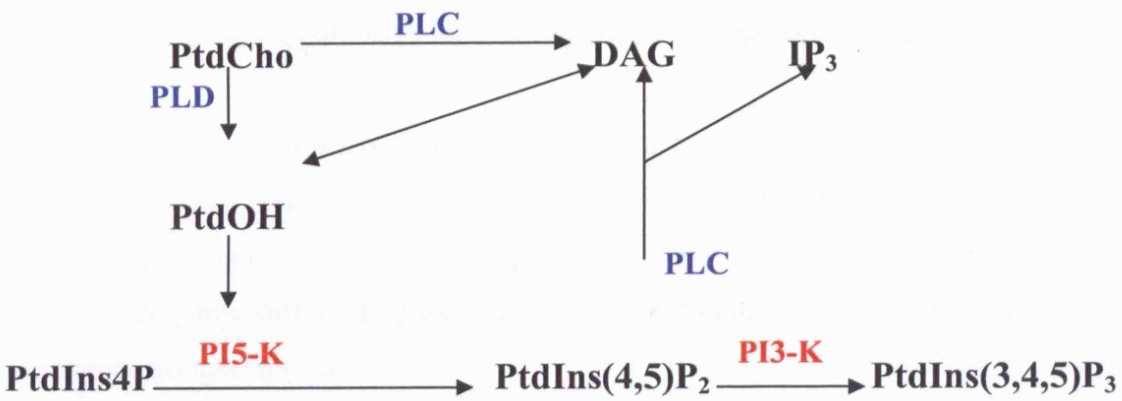


Figure 1:3 Illustrative examples of the actions of phospholipases (blue) and PI kinases (red).

1.5 Protein interactions

A single protein is often involved in multiple signal transduction pathways within a cell. This poses a problem since for an appropriate biological response to a signal to be achieved the information flow along a pathway must be correct and this requires each of the series of biochemical reactions being tightly controlled. To achieve this control the actions of enzymes such as kinases and phosphatases are regulated both temporally and spatially through interactions with both proteins and lipids to generate signalling complexes at distinct cellular locations. These interactions help bring together enzyme and substrate in an appropriate cellular location and so aid the flow of information along the signalling pathway.

The use of modular interaction domains provides a way in which these protein interactions can be controlled and signalling pathways integrated. Interaction domains tend to consist of independently folding modules of 35-150 amino acids that recognise

specific peptide or lipid motifs. Many proteins contain multiple interaction domains allowing them to bind to several partners at once and consequently increasing the diversity of signalling pathways they can operate in (reviewed by (Pawson and Nash, 2003)).

Some common protein interaction domains are the Src-homology-3 (SH3) and WW domains that both bind to proline rich regions. Domains that interact with lipids include the pleckstrin homology (PH), FYVE, phox homology (PX), and C1 domains. These domains recognise different lipids and are important in targeting proteins to specific membrane compartments.

Another group of interaction domains recognise particular post-translational modifications ((Seet et al., 2006) for review). Examples of these include Src-homology-2 (SH2) and phosphotyrosine-binding (PTB) domains which both bind to particular phosphorylated tyrosine sequences. Domains that bind to phospho-serine/threonine sequences include some WD40 repeats, 14-3-3 and Forkhead-associated (FHA) domains. Domains that recognise PTMs other than phosphorylation include the Bromodomain that binds to acetylated lysine residues in target proteins and the TUDOR domains that frequently bind to methylated lysine or arginine residues.

Increasingly it is becoming clear that some of these interaction domains although originally described as being either a protein or a lipid recognition domain are able to bind to both lipids and protein sequences (see (Balla, 2005) for review). This finding adds another level of complexity to the way in which protein complexes are targeted and formed to give rise to signal transduction.

1.6 The cytoskeleton and signalling

The cytoskeleton is a dynamic multiprotein structure that provides support and mechanical strength to cells. Through the association with a wide variety of proteins the cytoskeleton also acts as a mechanotransducer, organises organelles and participates in vesicle trafficking. The cytoskeleton consists of three types of filaments, these are microfilaments (MF), microtubules (MT) and intermediate filaments (IF). The subunit proteins of microfilaments and microtubules are actin and tubulin respectively, intermediate filaments however are made up a much more diverse range of proteins with subunits displaying cell type dependent expression (for review (Herrmann et al., 2007)). The cytoskeleton is regulated through the association of scaffolding and catalytic proteins (Juliano, 2002) that also serve to interlink the filaments and connect the cytoskeleton to the ECM and adjoining cells through adhesions such as desmosomes, focal contacts and focal adhesions. Increasingly these contacts through which the cytoskeleton is linked to other cells and the ECM are being seen as important in allowing the cell to convert mechanical forces into biochemical signalling (Katsumi et al., 2004). Many cells are exposed to physical forces, for example, endothelial cells undergoing shear stress and cells exposed to hyperosmotic/hypotonic conditions are subjected to mechanical force as a result of cell volume alterations. New techniques using optical tweezers have allowed the effect of the local application of force to be examined (Hormeno and Arias-Gonzalez, 2006). The ability of cells to sense force is demonstrated by the observations that cells grown on flexible substrates migrate faster than on more rigid substrates (Pelham and Wang, 1997). Despite the complex nature and regulation of the cytoskeleton a few key regulators have emerged and the best characterised of these are the Rho family GTPases, namely Rac1, RhoA and Cdc42.

1.6.1Rho GTPases

The Rho GTPases are a distinct family within the superfamily of Ras-related small GTPases and are encoded by twenty-two mammalian genes. They act as molecular switches cycling between an active GTP-bound form and an inactive GDP-bound form. Their activity is controlled by guanine nucleotide exchange factors (GEFs) that catalyse the exchange of GDP for GTP and GTPase activating proteins (GAPs) that inactivate these switches by stimulating their intrinsic GTPase activity. Many Rho GTPases undergo post-translational modifications such as phosphorylation and prenylation and also associate with guanine nucleotide dissociation inhibitors (GDIs). This combination of control mechanisms functions to localise and control their action and has been recently reviewed (Buchsbaum, 2007). It has been calculated that approximately 1% of the human genome encodes proteins that either regulate or are regulated by direct interaction with Rho GTPases (Jaffe and Hall, 2005). Rho, Rac and Cdc42 are the most studied of the Rho GTPases and so far over 50 effector proteins have been identified for them. The effectors are a diverse range of proteins and include serine/threonine kinases such as the PKNs (Amano et al., 1996b) (Vincent and Settleman, 1997), scaffold proteins for example WASP (Rohatgi et al., 1999) and lipid kinases such as PI3-kinase (Bokoch et al., 1996), (Tolias et al., 1995). Through this diverse range of effectors the Rho GTPases exert considerable control over cell processes (reviewed by (Jaffe and Hall, 2005)) such as polarity, cell division, vesicle trafficking and cell motility. Of particular interest to this thesis is the role the Rho GTPases play in regulation of the cytoskeleton.

Activation of Rho, Rac or Cdc42 results in distinct effects on the actin cytoskeleton. Rho activation leads to the assembly of actin/myosin contractile filaments, Rac and Cdc42 activation lead to the formation of protrusive actin rich lamellipodia and filopodia respectively (Etienne-Manneville and Hall, 2002). These differing effects on the actin cytoskeleton are brought about through distinct controls on actin polymerisation and organisation. Whilst Rac and Cdc42 both initiate actin polymerisation at the periphery of the cell through the Arp2/3 complex they do so through acting on complexes containing different members of the Wiskott-Aldrich syndrome (WASP) family proteins (Raftopoulou and Hall, 2004). The Arp2/3 complex

is heptameric and associates with the sides and perhaps also the ends of existing actin filaments to initiate the formation of new, branched filaments (Millard et al., 2004). Rho however initiates actin polymerisation through the activation of formins. The binding of formin dimers such as mDia1 to the plus end of actin filaments prevents the binding of capping proteins that normally terminate actin polymerisation and results in linear elongation of actin filaments (for review see (Goode and Eck, 2007)).
The newly formed actin filaments must also be correctly organised and in particular the branched filaments formed by Cdc42 must be organised into the unbranched bundles found in filopodia. Whilst the mechanism of this is as yet unclear it is fairly well defined that Rho-induced assembly of actin/myosin filaments is mediated through Rho-kinase (ROCK) inactivating myosin light chain (MLC) phosphatase. This leads to increased phosphorylation of MLC that promotes the actin filament cross-linking activity of myosin II (Kimura et al., 1996) (Amano et al., 1996a).
The Rho GTPases can also affect actin dynamics through their effects on cofilin that acts to sever and depolymerise actin filaments. Rho family GTPase effectors such as ROCK (Rho) and PAKs (Cdc42, Rac) activate LIM kinases that inactivate cofilin via phosphorylation and induce cytoskeletal rearrangements (Kuhn et al., 2000).

Rho GTPases can also affect microtubule dynamics which similar to actin filaments have intrinsic polarity with a minus end which is usually anchored to the centromere and a dynamic plus end which is usually located at the cell periphery. The maintenance of cell polarity is essential for directed cell migration and microtubule plus-end binding proteins such as CLIP-170 that are localised at the cell cortex play a major role in defining cell polarity and cell shape (for review see (Siegrist and Doe, 2007)). CLIP-170 can interact with the Rho GTPase effector IQGAP1 that has been shown to be required for cell motility (Mataraza et al., 2003). Recent work from our lab has demonstrated that PKN1 and PKN3 can phosphorylate CLIP-170 *in vitro* (Dr Collazos, personal communication), it remains to be seen what the *in vivo* significance of this is.

1.7 Signalling and disease

Given the multitude of physiological events that signal transduction pathways control and the complex interplay between components of these pathways it is perhaps not surprising that when a small element of a pathway malfunctions disease is often the result. Examples of diseases caused by aberrant cell signalling include diabetes and cancer. Since cancer is the focus of the work presented in the latter part of this thesis it is this disease that I will discuss in further detail.

1.7.1 Hallmarks of cancer.

It has been suggested that there are six essential alterations a cell must undergo to give rise to a malignant cancer (Hanahan and Weinberg, 2000). The gain of these characteristics is usually mediated through the hyperactivation of oncogenes and/or the inactivation of tumour suppressor genes. More than 100 proto-oncogenes and tumour suppressor genes have been identified thus far and their dysregulation affects multiple signalling pathways and consequently leads to inappropriate cell behaviour. The hallmarks of cancer, important oncogenes and tumour suppressors as well as a consideration of the therapies being developed based on our knowledge so far will now be considered.

1) Self-sufficiency in growth signals.

Cells are normally stimulated to proliferate in response to growth factors but cancer cells can proliferate independent of normal growth factor signalling. This ability can arise either from cells becoming able to produce growth factors, mutation or overexpression of growth factor receptors such as those of the EGFR family or alterations in components of the pathways downstream of these receptor (recently reviewed (Normanno et al., 2006)). These alterations can result in inappropriate activation of the proliferative pathway and hence uncontrolled cell growth.
Growth factor receptors are commonly overexpressed in cancers of the breast and therapies have been developed to target these receptors. For example 20-30% of breast

cancers express higher than normal levels of human epidermal growth factor receptor 2 (HER2) and this correlates with a more aggressive tumour type (Berger et al., 1988) (Guerin et al., 1988). Herceptin is a monoclonal antibody that recognises HER2 and is used in the treatment of HER2 positive tumours to inhibit HER2 mediated signalling events (Eisenhauer, 2001)

2) Insensitivity to growth-inhibitory signals.

In the opposite fashion to above, cells also receive signals that prevent them proliferating and for a cancer to develop these signals must be ignored. One of the key players in regulation of cell cycle progression is the retinoblastoma protein (pRb), so named because inactivation of both copies of the gene results in cancer of the retina (retinoblastoma). pRb negatively regulates cell cycle progression by binding to the transcription factor E2F and preventing the expression of genes required for a cell to progress through S phase. To proceed through the G1 checkpoint pRb is phosphorylated downstream of cyclinD1 activation. (reviewed in (Yamasaki, 2003). These regulatory controls are disrupted in the majority of tumours frequently through mutations in components of the pathway, loss of expression of pRb or overexpression of cyclinD1. A better understanding of the nature of the pRb/cyclin pathway in different cell types and its contribution to the development of different cancers will be critical if useful therapeutics are to be developed.

3) Evasion of programmed cell death (apoptosis).

Apoptosis is essential to maintain a homeostatic balance of cell numbers and to prevent cells containing damaged DNA dividing and passing on potentially harmful mutations. The tumour suppressor p53 is activated in response to DNA damage and plays a key role in arresting the cell cycle to allow time for the DNA damage to be repaired and promoting apoptosis if the DNA damage is too great. Without functional p53 signalling a cell can accumulate mutations that will potentially contribute to neoplastic growth. The importance of p53 as a tumour suppressor is illustrated by the fact that it is defective in at least 50% of human tumours (Levesque and Eastman, 2007).

Targeting p53 for therapeutic gain in the treatment of cancer has been the focus of much research and particular focus has been placed on small molecule compounds that would reactivate p53 e.g. PRIMA-1 and CP-31398. As yet though no treatments have made it to the clinic (Wiman, 2006).

4) Limitless replicative potential.

Although the processes described thus far would seem to be enough for a programme of continuous cell growth it has been found that most if not all cells can only undergo a certain number of cell divisions prior to arriving at a state of senescence (i.e. no more replication). This is due to the shortening of the chromosome ends that occurs with every cell cycle reaching a critical point where any subsequent shortening due to more cell cycles would result in damage to gene encoding areas of the chromosome (for review see (Hathcock et al., 2005)). However in approximately 85% of human cancers telomere length is maintained (Kelland, 2007). The majority of these cells appear to achieve this through high levels of expression of the telomerase enzyme and the result is that the cells are effectively immortal. The most advanced telomerase specific therapy is GRN163L, an oligonucleotide based molecule that act as a telomerase RNA template antagonist and is currently in phase I clinical trials.

5) Sustained angiogenesis.

The formation of new blood vessels is critical if a tumour is to grow above 1-2mm in size, this is because the diffusion of oxygen and nutrients can only occur over a short distance (Hanahan and Folkman, 1996). The best understood angiogenesis signal is that of the production of vascular endothelial growth factor (VEGF) by neoplastic cells. Surrounding endothelial cells express receptors for VEGF and tumour produced VEGF stimulates the formation of new blood vessels that supply the tumour with the oxygen and nutrients necessary for further growth (Roy et al., 2006).
Bevacizumab is a monoclonal antibody that has been licensed for use in the treatment of colon cancer. Bevacizumab works by binding to VEGF and stopping it from binding to the VEGF receptors so inhibiting the signalling required for new blood vessels to form.

Small molecule inhibitors of the VEGF receptor tyrosine kinases are also being developed to try and block the signalling pathways required for angiogenesis (de Gramont and Van Cutsem, 2005).

6) Tissue invasion and metastasis.

The ability of tumour cells to break away from the primary tumour site and spread to other sites in the body is arguably the most important in terms of prognostic outcome for a patient (Sporn, 1996). Whilst a benign tumour can grow so large that it disrupts the normal functioning of surrounding tissues and organs they can often be surgically removed and usually will not return. However the ability of a neoplastic cell to migrate out of the primary tumour bulk, invade the extracellular matrix and travel to other regions in the body where they can form new tumours makes these metastatic cancers hard to treat.

The mechanisms behind tumour cell invasion are not clearly defined but the loss of cell-cell adhesion appears important as is demonstrated by the loss of E-cadherin expression in many aggressive tumour types (Christofori and Semb, 1999). Additionally degradation of the surrounding matrix and cell surface receptors by proteases is implicated in the ability of cells to move out of the primary tumour site, see (Yoon et al., 2006) for a review . In particular matrix metalloproteinases (MMPs) are often upregulated in malignant tumours but although initial studies on MMP inhibitors were promising the results from human clinical trials were disappointing (Coussens et al., 2002). Therefore focus has again turned to understanding the molecular mechanisms of cancer cell motility to guide the development of novel therapeutics.

1.7.2 Cell motility

Cell motility is important both physiologically, for example in development and in migration of immune cells to sites of infection, and pathologically where it results in the spread of cancer cells to sites distal to the primary tumour (metastasis). Cell motility occurs in several distinct processes that nonetheless have some common features (Raftopoulou and Hall, 2004). Chemotaxis is the movement of cells towards a chemical signal such as a neutrophil moving towards cytokines released at sites of infection. Invasion is the process by which cancer cells move through the surrounding ECM, this involves production of proteases to degrade the matrix of proteins. *In vitro* descriptions of motility distinguish between movement in 2-dimensions along a flat surface (migration) and 3-dimensions through a matrix of proteins (invasion).

Despite the different types of cell motility they all have some common requirements to achieve forward movement (reviewed in (Friedl and Brocker, 2000)), these are:

1) Extension of a leading edge.

2) Formation of new sites of adhesion to the ECM.

3) Cell body contraction.

4) Detachment of adhesions at the rear of the cell.

To achieve these events requires maintenance of cell polarity as well as dynamic coordination of focal adhesions and the cytoskeleton to produce the force necessary to drive the cell forwards. Although the precise regulation of these processes depends both on cell type and the microenvironment some of the general mechanisms that have emerged nicely illustrate the concepts of signal transduction previously introduced.

Rac and Cdc42 are both primarily thought to be required at the front of the cell where they promote the formation of lamellipodia and filopodia respectively (Nobes and Hall, 1995) through the localised polymerisation of actin (see section 1.6.1). One mechanism of Rac and Cdc42 activation at the leading edge is thought to be through the action of PI3-kinases stimulated by ligand binding to cell surface receptors. PI3-kinase activation

results in localised production of PtdIns(3,4,5)P_3 and recruitment of Rac-GEFs containing lipid interaction domains such as PH domains (Welch et al., 2003). Concurrent with Rac mediated formation of a leading edge, nascent focal complexes must also be formed to provide a link between the ECM, integrins, the cytoskeleton and downstream intracellular signalling events. Multiple tyrosine phosphorylation events are observed at sites of focal complex formation. The major contributors to this are focal adhesion kinase (FAK) and Src family kinases (Mitra and Schlaepfer, 2006). Integrin activation results in FAK binding to the cytoplasmic tails of integrins via its FAT (focal adhesion targeting) domain where it becomes phosphorylated at Y397. This creates a binding site for Src that then further phosphorylates FAK and other associated proteins. FAK contains multiple protein interaction domains that act to recruit SH2 and SH3 domain containing proteins such as p130Cas, paxillin and Grb2. These signalling complexes can then also activate Rac through recruitment of the bipartite Rac-GEF ELMO1/Dock180 (Lu and Ravichandran, 2006). Interestingly high expression levels of ELMO1 and Dock180 have recently been found in invading glioma cells (Jarzynka et al., 2007). The co-ordinated actions of Src and FAK are important in regulating the formation of focal adhesions both temporally and spatially. This is illustrated by the observations that $FAK^{(-/-)}$ cells have enhanced focal adhesions and impaired migration (Ilic et al., 1995) whereas transformation of cells by Src is associated with decreased adhesion and increased migration (Platek et al., 2004). Phosphatases such as PTP-PEST are also involved in the signalling events downstream of integrin activation to enable fine control over activities and interactions of the various proteins involved in regulating migration (see (Larsen et al., 2003) for review).

The maturation of focal complexes to the larger focal adhesions found towards the centre of a migrating cell and production of the contractile force required to retract the trailing edge is attributed primarily to the action of Rho. Rho acts through its effector, Rho kinase (ROCK), to increase contraction of actin:myosin filaments by increasing the phosphorylation of myosin light chain both directly (Amano et al., 1996a) and by inhibition of myosin light chain phosphatase (Kimura et al., 1996). The mechanism by which Rho activity is suppressed at the leading edge and upregulated towards the centre and rear of migrating cells is not clear but is likely to be controlled through the temporal

and spatial control of Rho-GEFs and Rho-GAPS such as p190RhoGAP (Grande-Garcia et al., 2007).

Whilst the imaging of cell motility has traditionally been carried out on cells migrating in 2-dimensions it has become increasingly clear that there are fundamental differences between 2-D and 3-D cell motility. In order for a cancer to metastasise tumour cells must cross tissue barriers and move in 3-dimensions. This requires regulation of the ECM and proteases such as MMPs that degrade ECM components were thought to be essential to allow tumour cells to invade. However imaging of cells moving through a 3-D matrix has revealed that tumour cells are capable of moving through a matrix without degrading the matrix. This was shown to be dependent on Rho/ROCK signalling and cells had a round morphology with membrane blebs. However cells were also capable of moving through the matrix with an elongated shape and this required Rac rather than Rho activity and the action of extracellular proteases (Wyckoff et al., 2006) (Sahai and Marshall, 2003). These findings demonstrate the complex nature of cell motility regulation.

1.8 Protein kinase C

The importance of kinases in the regulation of signal transduction has been illustrated above. A family of Ser/Thr kinases that has diverse roles to play in the regulation of many physiological processes is the Protein kinase C (PKC) family. They have been reported to have a bewildering array of functions and it is most likely that although there is some redundancy between isoforms the tissue specific expression dictates the relative role each isoform plays in any given process. (reviewed by (Mellor and Parker, 1998)). The family consists of 12 distinct genes although alternative splicing gives rise to a greater number of proteins. They are characterised by their highly conserved catalytic domains and grouped into sub classes based on their regulatory domains. The single *S.cerevisiae* PKC isoform, pkc1, contains all the conserved domains that are associated with PKC regulation and mammalian PKC isoforms contain selections of these domains (see Figure 1:4).

The classical PKCs include the α, β, and γ isoforms that are activated by phosphatidylserine in a calcium dependent manner and are also activated by DAG. The novel PKC class consists of δ, θ, ε, and η isoforms and are activated by DAG. The ι and ζ isoforms make up the atypical PKC class and these are both calcium and DAG independent. The most recently identified family members make up the Protein kinase N (PKN) subfamily and are also known as protein kinase C related kinases (PRKs) (for review see (Mukai, 2003)). The PKNs consist of at lease three isoforms (PKN1, PKN2 and PKN3) which all have distinct regulatory domains to those of the other PKCs. They are activated independently from calcium and DAG but are able to be activated by members of the Rho family of small GTPases.

The differing modes of regulation are determined by the domains found in the N-terminal regulatory domain of the PKCs (for a review see (Corbalan-Garcia and Gomez-Fernandez, 2006)). For example the C1 domain of the cPKCs and nPKCs contribute to lipid responsiveness and targets PKCs to membranes through its ability to bind PtdSer and DAG. The C1 domain of the aPKCs however contain only a single zinc-finger like motif (the cPKCs and nPKCs contain two) and are not responsive to DAG.

A C2 or C2-like domain is found in all PKC isoforms. The C2 domain is a calcium binding domain whereas the C2-like domain does not bind calcium, this explains the relative calcium dependence or independence of the PKC classes. The C2 domain is also found in other proteins such as synaptotagmin and have been shown to mediate protein-protein interactions (Ponting and Parker, 1996). The V3 region represents a flexible hinge between the regulatory and catalytic domains that varies considerably between PKC isoforms. It is therefore likely that this region contributes to some isoform specific functions through distinct protein-protein interactions as has been demonstrated in the case of the PKNs (see 1.8.1).

The catalytic domains of PKC family members have a classical bilobal kinase structure as seen in the crystal structures of PKCθ and PKCι (Xu et al., 2004) (Messerschmidt et al., 2005). In order for the kinase to be maximally active a residue within the kinase domain known as the activation loop must be phosphorylated (T-loop phosphorylation) and in most cases this seems to be achieved through the action of PDK1 (Le Good et al.,

1998) (Flynn et al., 2000). Isoform specific modes of regulation therefore ultimately control the activity of the kinase through this activation loop phosphorylation that is thought to be needed to correctly align catalytic active site residues (Cazaubon and Parker, 1993).

C-terminal to the catalytic domain is another variable region termed the V5 region. This region contains two further phosphorylation sites that are thought to play a role in maintaining PKCs in a stable conformation (Bornancin and Parker, 1997). The TP or turn motif is conserved in all PKC family members whereas the hydrophobic site or FSY motif is absent in the aPKCs and PKNs. In these PKC family members the serine is replaced by an acidic residue (glutamate in the aPKCs and aspartate in the PKNs), these substitutions may however mimic phosphorylation. The identity of the kinases responsible for phosphorylation of the TP and FSY sites are the subject of much speculation and are often thought to be effected by autophosphorylation.
It has been reported recently that the very C-termini of PKCε and PKN1 are critical for catalytic activity and stability (Zhu et al., 2006) (Lim et al., 2006). In the case of PKN1 the last seven amino acids were reported to be essential for full lipid responsiveness, stability and full activity (Lim et al., 2006) (Lim et al., 2005). The V5 region although only approximately 50 amino acids long therefore still plays a vital role in regulation of the PKCs. This is perhaps best illustrated by the different functions of the two PKCβ splice variants that only differ in this V5 region. This region must therefore be responsible for any differences observed between them, for example in U937 monocytic cells $PKC\beta_I$ localises to microtubules whereas $PKC\beta_{II}$ is found in secretory granules (Kiley and Parker, 1995).

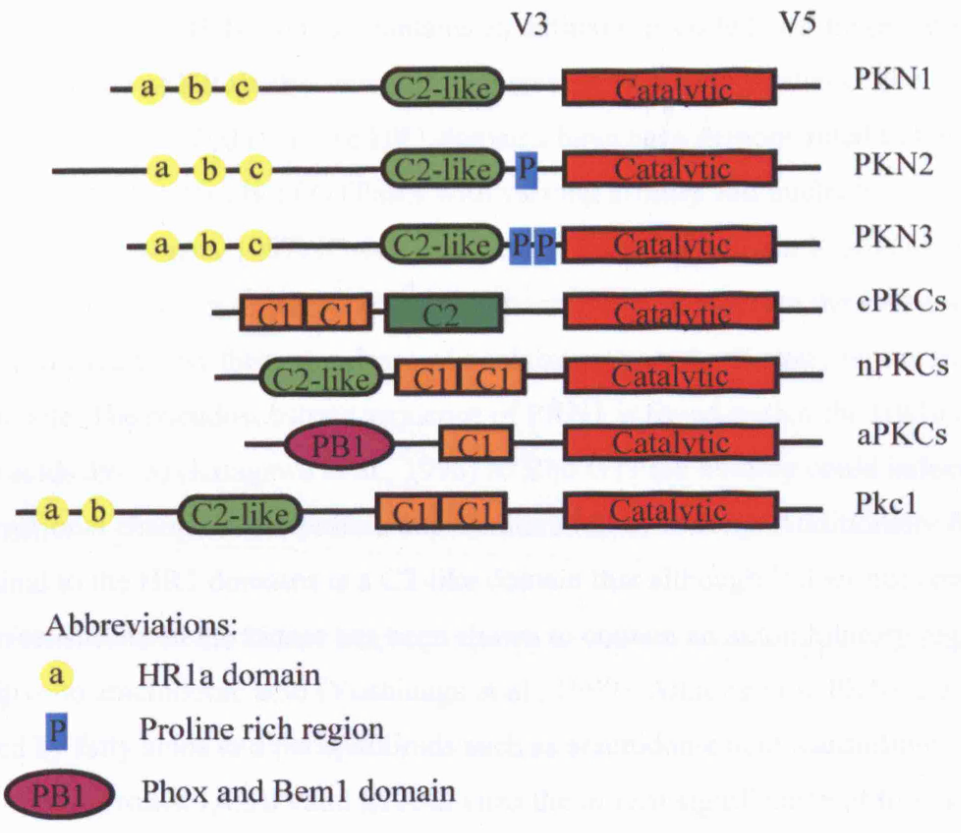


Figure 1:4 Schematic representation of PKC superfamily domain structures.

1.8.1PKN subfamily

The PKNs were identified 13 years ago based on their homology to the PKCs (Mukai and Ono, 1994) (Palmer et al., 1994). So far three isoforms have been identified PKN1 (PRK1/PKNα), PKN2 (PRK2/PKNγ) and PKN3 (PKNβ). PKN1 and PKN2 are expressed in many tissues (Mukai and Ono, 1994) (Quilliam et al., 1996), PKN3 meanwhile was undetected in adult tissues but is expressed abundantly in cancer cell lines (Oishi et al., 1999). Their catalytic domains are highly homologous to those of other PKC family members as has already been discussed, but they are regulated through distinct mechanisms.

Each of the three isoforms contain three repeated domains termed the homology region 1 (HR1a,b,c) or ACC domain (for antiparallel coiled-coil fold). These regions are approximately 70 amino acids long and each contain a leucine zipper like sequence. The X-ray structure of HR1a in complex with RhoA has been solved (Maesaki et al., 1999)

and showed that the HR1a domain contains an antiparallel coiled-coil finger domain, the solution structure of HR1b also revealed the presence of an antiparallel coiled-coil domain (Owen et al., 2003). These HR1 domains have been demonstrated to bind members of the Rho family of GTPases with varying affinity and nucleotide dependence (Vincent and Settleman, 1997) (Owen et al., 2003) (Watanabe et al., 1996) (Amano et al., 1996b). The binding of Rho GTPases has been shown to activate the PKNs and this may be achieved partly through release of a inhibitory pseudosubstrate region from the catalytic site. The pseudosubstrate sequence of PKN1 is found within the HR1a domain (amino acids 39-53) (Kitagawa et al., 1996) so Rho GTPase binding could induce a conformational change in the protein and an alteration of activity. Additionally further C-terminal to the HR1 domains is a C2-like domain that although it does not confer calcium sensitivity to the kinase has been shown to contain an autoinhibitory region that is sensitive to arachidonic acid (Yoshinaga et al., 1999). Although the PKNs can be activated by fatty acids and phospholipids such as arachidonic acid, cardiolipin, PtdIns(4,5)P_2, PtdIns(3,4,5)P_3 and LPA *in vitro* the *in vivo* significance of this is unclear (Mukai et al., 1994) (Peng et al., 1996) (Morrice et al., 1994) (Palmer et al., 1995).
PDK1 has been shown to activate PKN1/2 via phosphorylation of its activation loop site *in vitro* and *in vivo* (Dong et al., 2000) (Flynn et al., 2000). Rho was shown to control the interaction between PKN1 and PDK1 in endosomes this interaction was independent of PI3-kinase but activation loop phosphorylation of PKN1 was inhibited by PI3-kinase inhibition (Flynn et al.).
PKN1 has also been shown to be cleaved in response to apoptosis in cultured cells (Takahashi et al.) and after ischemia/reperfusion induced apoptosis of rat retinas (Sumioka et al.). In both cases PKN1 was found to be cleaved and a catalytic fragment corresponding to the catalytic domain generated. This adds another mechanism by which PKN1 is activated however it is not known whether the other PKN isoforms are similarly activated by apoptotic induced cleavage.

One of the most striking differences between the PKN isoforms is the presence of one or two proline rich regions in the V3 regions of PKN2 and PKN3 respectively. These regions have been shown to mediate binding of SH3 domain containing proteins. In the

case of PKN2 the scaffolding proteins Nck and Grb4 have been shown to interact with this region (Quilliam et al.) (Braverman and Quilliam). Nck and Grb4 both contain an SH2 domain that enables them to bind to Tyr phosphorylated residues of kinase receptors in response to specific signals such as growth factors (Buday et al., 2002). PKN3 meanwhile has been shown to bind GRAF (GAP for Rho associated with focal adhesion kinase) and the related protein GRAF2 (Shibata et al.). The V3 region of PKN1 although it lacks a proline rich region also mediates protein interactions and has been shown to interact with TRAF2 (TNFα receptor associated factor 2) and contribute to activation of NFκB signalling (Gotoh et al., 2004). Isoform specific protein-protein interactions such as these therefore contribute to the assembly of distinct signalling complexes in response to different upstream signals.

Like other kinases the regulation of PKN activity must be tightly controlled both temporally and spatially. For example the PKNs have been shown in several contexts to play a role in controlling the cell cycle and in particular the timing of the transition from G2 to M phase. In *Xenopus* cycling egg extracts and vascular smooth muscle cells (VSMCs) PKN1 phosphorylates the phosphatase Cdc25C. This results in an inhibition of Cdc25C activity and a delay to mitosis due to a delay in the Cdc2/cyclinB1 complex being activated by dephosphorylation. In the VSMCs this delay in mitosis was mediated by Rho and PKN1 and occurred in response to TGFβ1 treatment (Su et al.) (Misaki et al.). In HeLa S3 cells PKN2 was shown to be regulated by Rho GTPases and control both entry into mitosis and abscission of the midbody to complete cytokinesis. In this system PKN2 was required to phosphorylate and activate Cdc25B, the phosphatase required to activate the cyclin/Cdk1 complex and promote G2 to M progression (Schmidt et al., 2007).

An example of the spatial control of a PKN isoform comes from work in our lab that demonstrated that ectopically expressed PKN1 translocated in a reversible manner to punctate structures in response to hyperosmotic stress, this behaviour was dependent on Rac1. PDK1 was also recruited to these structures and consistent with this PKN1 was seen to undergo activation loop phosphorylation in response to hyperosmotic stress.

Sensing the osmolarity of the environment is critical for all organisms and they have evolved mechanisms to cope with either hyper or hypo osmotic conditions (reviewed in (Bourque et al., 1994)). Changes in osmolarity are detected by osmosensors that then activate a variety of signalling cascades. Protein families activated in response to hyperosmolarity include members of the Rho GTPases (Di Ciano et al., 2002) (Di Ciano-Oliveira et al., 2003), the MAPkinases (Galcheva-Gargova et al., 1994), (Han et al., 1994) (Matsuda et al., 1995), PKCs (Zhuang et al., 2000) and src family kinases (Reinehr et al., 2004), (Volonte et al., 2001). In contrast to PKN1, PKB has been shown to be down-regulated in response to hyperosmolarity (Meier et al., 1998). These signalling pathways cause a variety of rapid post-translational and slower transcriptional changes that help restore cell volume, reinforce the cytoskeletal architecture and delay the cell cycle. All these adaptations help cells to survive transient increases in osmolarity.

As a consequence of hyperosmolarity changes in gene expression occur, for example the activation of the transcription factor OREBP allows accumulation of compatible osmolytes that help restore cell volume (Ko et al., 2000). Interestingly there have been several reports of PKN1 controlling transcriptional activity. PKN1 has been shown to interact with the NDRF/NeuroD2 transcription factor (Shibata et al., 1999) and to activate promoters responsible for smooth muscle cell differentiation in response to TGF-β (Deaton et al., 2005) or starvation (Cottone et al.). PKN1 was also shown to translocate from the cytoplasm to the nucleus in response to heat stress suggesting a role for PKN1 in control of transcriptional responses under this stress condition (Mukai et al., 1996a). Additionally PKN1 has been shown to induce transcriptional activation of the androgen receptor (Metzger et al.) and act downstream of Rho to drive activity of the serum responsive element of the c-fos gene promoter (Morissette et al.) and the c-jun promoter (Marinissen et al., 2001).

The PKN1 dependent effects on c-jun gene expression were shown to be mediated through a signalling pathway involving MKK3/MKK6 and p38γ hence linking PKN1 to regulation of this mitogen activated protein kinase (MAPK) signalling cascade. Further linking PKN1 to the p38 pathway is the finding that PKN1 is able to phosphorylate MLTKalpha (MLK like mitogen activated triple kinase), a MAPK kinase kinase for the

p38 pathway. Interestingly the expression of dominant negative PKN1 inhibited the phosphorylation of MLTKalpha in response to hyperosmotic shock (Takahashi et al.).

Another consequence of hyperosmolarity is remodelling of the cytoskeleton that presumably occurs to help cells withstand the mechanical stress that results from cells shrinkage due to an efflux of water. Changes in the cytoskeleton include an increase in F actin content and organisation mediated at least in part by Rac and Cdc42 (Di Ciano et al., 2002). Prehaps not surprisingly considering their control by Rho GTPases the PKNs have been linked numerous times to the cytoskeleton. PKN1 has been shown to interact with α-actinin *in vivo* and this interaction was stimulated by PtdIns(4,5)P_2 *in vitro* (Mukai et al.). Endogenous α-actinin and PKN1 were also shown to interact in platelets and this interaction is greatly increased in response to pathological shear stress (Feng et al.). Further links between PKNs and the dynamic actin cytoskeleton have been made through overexpression studies. Expression of wild type PKN1 was found to induce membrane ruffling and actin cytoskeleton reorganisation mimicking the rearrangements seen in response to insulin stimulation. Expression of kinase dead forms of PKN1 however prevented the rearrangements of the actin cytoskeleton usually observed in response to insulin stimulation (Dong et al.). This is particularly interesting since hyperosmolarity, like insulin stimulation, induces phosphorylation of the insulin receptor (Ouwens et al., 2001). PKN1 has also been implicated downstream of Rho in regulating GLUT4 dynamics and glucose uptake in response to insulin (Standaert et al., 1998) and so it appears that PKN1 may have roles to play in both insulin and hyperosmotic induced signalling pathways. Further evidence for PKN regulation of the cytoskeleton comes from reports that PKN1 is able to interact with and phosphorylate the head domains of the intermediate filament proteins vimentin, glial fibrillary acidic protein (GFAP) and neurofilaments *in vitro* and so inhibit filament formation (Matsuzawa et al.) (Mukai et al., 1996b).

Previously the importance of regulation of the cytoskeleton to achieve cell motility was discussed and perhaps not surprisingly given their status as Rho GTPase effectors PKNs have been implicated in elements of cell motility. The activation of Rac1 by the ECM constituent hyaluronan (HA) binding to the CD44 receptor results in activation of PKN2

and enhanced migration of astrocytes (Bourguignon et al.). PKN2 has also been shown to bind to the transmembrane phosphatase PTB-BL that has been linked to cytoskeletal rearrangements and they were found to colocalise in lamelliopodia structures (Gross et al., 2001). Lamellipodia must be dynamically regulated to enable cell migration and it is possible that the PKN2 interaction with PTB-BL helps achieve this regulation. The regulation of cell-cell adhesions is essential for the proper establishment and maintenance of organised tissues, and pathologically the ability of cells to disrupt contacts with neighbouring cells is associated with greater metastastic potential. PKN2 has been implicated in the control of cell-cell adhesion in keratinocytes by operating downstream of Rho and inducing phosphorylation of Fyn and catenins to enhance adhesion (Calautti et al.). The role of PKN2 in cell migration therefore appears to depend on the cell type examined since the formation of strong cell-cell adhesions is generally associated with decreased adhesion whereas in the astrocyte model PKN2 activity enhanced migration. PKN3 has been shown to be required for the metastasis of PC3 cells in an orthotopic mouse tumour model implicating a role for PKN3 in controlling cell invasion (Leenders et al.).

In conclusion it is apparent that the PKNs are important regulators of cell behaviour that participate in multiple signalling pathways. Interactions with protein and lipid partners regulate their participation in signalling downstream of specific signals by controlling both their location and activity within the cell. The PKNs status as Rho GTPase effectors links them to control of the cytoskeleton and a role for the PKNs in cell motility is just beginning to emerge with PKN2 and PKN3 implicated in migration and invasion respectively. This work seeks to further our knowledge both of the spatial control and functional roles of PKN1.

Chapter 2

Materials and Methods

2 Materials

2.1 Chemicals and plasticware

PfuTurbo DNA Polymerase was from Stratagene.

Optimem was from Gibco.

NuPAGE Bis-Tris gels, Lipofectamine 2000, Agarose was from Invitrogen.

HiPerFect was from Qiagen.

Ethanol and Methanol were from Fisher.

Transwell inserts with 8μm pores were from Corning.

BD Biocoat Matrigel Invasion chambers were from BD Biosciences.

T4 DNA ligase and restriction enzymes were from NEB.

Rainbow protein markers, hyperfilm, ECL and ECLplus were from Amersham-Pharmacia.

Phalloidin-Alexa546 was from Molecular Probes,

PVDF membrane was from Millipore.

MOWIOL 4-88 was from Calbiochem.

HPLC grade toluene, chloroform, methanol and water were from Rathburn Chemicals Ltd.

Phosphoinositides: PI(3)P (diC16, H^+), PI(4)P (diC16, H^+), PI(5)P (diC16, H^+), $PI(3,4)P_2$ (diC16, H^+), $PI(3,5)P_2$ (diC16, H^+), $PI(4,5)P_2$ (diC16, H^+) and $PI(3,4,5)P_3$ (diC16, H^+) were from Cell Signals. diC16 PtdIns (monosodium salt) was from Echelon Biosciences Inc.

Phospholipids: 1,2-dilauroyl-sn-glycero-3-phosphocholine (DPLC), 1,2-dilauroyl-sn-glycero-3-phosphoethanolamine (DPLE), 1,2-dilauroyl-sn-glycero-3-[phospho-L-serine] (sodium salt) (DPLS), 1,2-dilauroyl-sn-glycero-3-phosphate (monosodium salt)

(DPLA), 1,2-dilauroyl-sn-glycero-3-[phospho-rac-(1-glycerol)} (sodium salt) (DPLG) were from Avanti Polar Lipids Inc.

All other reagents were purchased from Sigma-Aldrich.

All glassware supplied by CR-UK research services except 5ml chromacol screw-top vials and specimen tubes (soda glass with polyethylene stoppers) were from VWR International.

Plasticware was purchased from Eppendorf, Corning, BD Falcon, Sterilin and Scientific Specialities.

2.2 Buffers

2 x LDS sample buffer

50% (v/v) 4 x LDS sample buffer (Invitrogen).

MOWIOL mounting solution

10% (w/v) Mowiol 4-88, 25% (v/v) glycerol, 100mM Tris pH 8.5. Incubate for 10 hrs at 50°C and mix thoroughly.

LB (Agar)

Provided by CR-UK research services. 10g/l Bacto-Tryptone, 5g/l yeast extract, 10g/l NaCl (6g agar/400ml LB).

PBS (Ca^{2+}/Mg^{2+} free)

Provided by CR-UK research services 8g/l NaCl, 0.25g/l KCl, 1.43g/l Na_2HPO_4, 0.25g/l KH_2PO_4, pH 7.2

TBS-T

20mM Tris pH 7.5, 0.9% (w/v) NaCl, 0.1% (v/v) Tween-20.

TAE

0.04M Tris, 0.1% glacial acetic acid, 1mM EDTA pH 8

Transfer buffer

20mM Trizma base, 200mM Glycine, 10% MeOH.

5 x Trypsin/Versene

Provided by CR-UK research services. 0.25% trypsin in Versene, pH 7.2: filter sterilised.

DMEM

Dulbecco's MEM (DMEM) was from CR-UK research services and was endotoxin free.

2.3 Antibodies

Name	Source	Antigen	Description	Use	Dilution
PKN1	Transduction Laboratories	PKN1	Mouse monoclonal	Immuno-blot (IB)	1:1000
PKN2	Transduction Laboratories	PKN2	Mouse monoclonal	IB	1:1000
PKN3	In house	PKN3	Rabbit monoclonal	IB	1:200
GFP-3E1	CR-UK	GFP	Mouse monoclonal	IB	1:1000
Tubulin	Sigma	Tubulin	Mouse monoclonal	IB	1:5000
Mouse-HRP	Amersham	Mouse IgG	HRP-linked secondary	IB-2° antibody	1:5000
Rabbit-HRP	Amersham	Rabbit IgG	HRP-linked secondary	IB-2° antibody	1:5000
AlexaFluor-488 (Donkey)	Molecular Probes	Mouse IgG	Flourescent secondary	Immunoflouresence	1:500

2.4 Cell types

MDAMB-468, MDAMB-231, MCF7, A459, CALU-1, HI299, PC3, LNCAP, COLO741, HT29, COLO320HSR and HEPG2 human transformed cell lines and NIH3T3 cells were from CR-UK cell production services.

786-0, A-498, Caki-1, SK-HEP-1, ACHN transformed human cell lines were obtained from the ATCC. GFP, GFP-PLD1a, GFP-PLD1b and GFP-PLD2 expressing stable polyclonal MDAMB-468 cells were generated by selection of expressing cells post-transfection with 100μg/ml G418. Resistant cells were then sorted using a FACS sterile sort to separate green cells and these were continued in culture in the presence of 500μg/ml G418.

Cells were maintained under conditions recommended by CR-UK cell production services but typically were split 1:4 every 3 days and maintained in DMEM + 10% FCS.

2.5 Constructs

Name	Source	Expression vector
GFP-PKN1	Dr Neil Torbett (Torbett et al., 2003)	pEGFP-C1 (Clontech)
DsRed-PKN1	Dr Neil Torbett (Torbett et al., 2003)	pDS-RED-C1 (Clontech)
GFP-PKN1 CD	See section 2.6.1.8	pEGFP-C1
GFP-PKCζ CD	See section 2.6.1.8	pEGFP-C1
GFP-Chimera 1	See section 2.6.1.8	pEGFP-C1
GFP-Chimera 2	See section 2.6.1.8	pEGFP-C1
GFP-Chimera 3	See section 2.6.1.8	pEGFP-C1
GFP-Chimera 2.1	See section 2.6.1.8	pEGFP-C1
GFP-Chimera 2.2	See section 2.6.1.8	pEGFP-C1
GFP-Chimera 2.3	See section 2.6.1.8	pEGFP-C1
GFP-49aa	See section 2.6.1.8	pEGFP-C1

2.6 Methods

2.6.1 Molecular biology

2.6.1.1 PCR reactions

Polymerase chain reactions (PCRs) were performed using a DNA Engine DYAD (MJ Research). The reaction mix contained: 1U Pfu Turbo, 1 x Pfu buffer, 0.4mM ultrapure deoxynucleotide triphosphate mix (0.4mM of each dNTP), 5% DMSO, 1μM sense primer, 1μM antisense primer and 100ng DNA template. Reactions were made up to 50μl in distilled water. PCRs were carried out using the following programme:
Step 1: 95°C, 2 mins.
Step2: 95°C, 45 secs.
Step 3: (Lowest primer Tm -4) °C, 45 secs.
Step 4: 72° C, (length of product in bps/500)mins.
Step 5: Cycle back to Step 2 x 35.
Step 6: 4°C, forever.

PCR products were examined by agarose gel electrophoresis and purified using the QIAquick gel extraction kit (Qiagen).

2.6.1.2 Agarose gel electrophoresis

Samples were 5:1 in 6 x DNA loading buffer and separated on a 1% agarose gel in 1 x TAE buffer alongside 1kb ladder DNA markers. 4% (v/v) ethidium bromide was added to the gel to allow visualisation of DNA on a transilluminator. Bands were excised and DNA extracted and purified for subsequent cloning using the QIAquick gel extraction kit (Qiagen).

2.6.1.3 Restriction digests

Restriction digests were carried out following the manufacturers guidelines.

2.6.1.4 Ligation reactions

Ligation reactions were performed using T4 DNA ligase . The reaction mix contained 400U T4 DNA ligase, 1 x ligase buffer, 100ng digested vector and insert DNA. Typically approximately a 3:1 molar ratio of insert:vector was used and a "vector only" control carried out in parallel. Ligation reactions were performed for at least 1 hr at 16°C.

2.6.1.5 Transformation of E.coli

Chemically competent DH5α or RapidTrans TAM1 (Active Motif) *E.coli* were transformed with purified plamids or ligation reaction products by heat shock. 50µl of competent cells were mixed with 100ng plasmid DNA or 3µl ligation reaction and incubated on ice for 30 mins. Cells were heat shocked at 42°C for 45 secs and then placed on ice for 2 mins. 500µl LB was added and the transformation mixtures were incubated with shaking at 37°C for 1 hr. Cells were plated onto LB plates containing the appropriate selection antibiotic (100 µg/ml ampicillin or 25 µg/ml kanamycin). Plates were inverted and incubated at 37°C overnight.

2.6.1.6 Plasmid DNA preparation

A colony from transformed DH5α cells was picked and grown overnight in a 5ml culture of LB containing the relevant selection antibiotic. From this small scale DNA preparations were carried out using the Qiagen mini-prep kit. For large scale DNA purifications a 5ml overnight culture was used to inoculate a 200ml culture and after another overnight incubation DNA was prepared using the Qiagen plasmid maxi-prep kit. The concentration and quality of DNA produced was measured using Nanodrop ND-1000 UV-Vis Spectrophotometer. The quality of the DNA was considered acceptable if the OD_{260} /OD_{280} ratio was in the range 1.6-1.8.

2.6.1.7 DNA sequencing

DNA sequencing was carried out using the ABI BigDye Terminator v3.1 system and following the manufacturers protocol. The reaction mix typically comprised 150ng plasmid, 3.2pmol primer and 1 x BigDye Terminator Mix in Sequencing Buffer (supplied by CR-UK services) made up to a final volume of 20µl in distilled water. Cycle sequencing was performed using the following programme:

Step 1: Ramp to 96°C at 2.5 °C/sec.

Step 2: 96°C, 1 min.

Step 3: 96°C, 10 secs.

Step 4: Ramp to (primer Tm -3) °C at 1°C/sec.

Step 5: (primer Tm -3)°C, 5 secs.

Step 6: Ramp to 60°C at 1°C/sec

Step 7: 60°C, 4 mins.

Step 8: Cycle back to Step 3 x 24.

Step 9: 2°C, forever.

Reaction products were purified using DyeEx 2.0 Spin Kits (Qiagen) and sequenced by CR-UK services on an Applied Biosytems 3730 DNA Analyser. Sequencing results were opened with EditView software and analysed using the DNA Strider package and NCBI Blast.

2.6.1.8 Construction of GFP-PKN1/PKCzeta chimeras

GFP-PKN1 CD, GFP-PKCzeta CD, GFP-C1, GFP-C2 and GFP-C3 were generated by amplification of myc-tagged chimeras in pcDNA3.1/Hygro (+) previously created by Dr Iturrioz. Sequences were amplified using the following primers:

Sense: 5'-CCGCTCGAGCGATGCCGGAGCAGAAGCTGATATCC-3'

Antisense: 5'-TAGAAGGCACAGTCGAGG-3'

The sense primer incorporated a restriction site for Xho1 to allow the PCR products to be cloned into pEGFP-C1 (Clontech) between the Xho1 and Apa1 MCS sites. This

resulted in the fusion of an N-terminal GFP tag in frame with the myc tagged PKN1/PKCzeta chimeras.

GFP-C2.1 GFP-C2.2 and GFP-C2.3 were generated using a 2-step overlapping PCR cloning strategy, the external primers in all cases were as above and PCR products from the second round of PCR were cloned into pEGFP-C1 (Clontech) between the Xho1 and Apa1 MCS sites as before.
Internal primers were as follows:
GFP-C2.1:
Sense 5'-GTTACAGGGACCTGAAGTTGGACAACGTCCTCCTCGATGCC-3'
Antisense 5'-GGCATCGAGGAGGACGTTGTCCAACTTCAGGTCCCTGTAAAC-3'
GFP-C2.2:
Sense 5'-GCTGTGGACTGGTGGGGACTGGGTGTCCTTATGTTTGAG-3'
Antisense 5'-CTCAAACATAAGGACACCCAGTCCCCACCAGTCCACAGC-3"
GFP-C2.3:
Sense 5'-CGCTACCCCCGCTTCCTGTCGGTCAAGGCCTCACACGTCTTG-3'
Antisense 5'-CAAGACGTGTGAGGCCTTGACCGACAGGAAGCGGGGGTAGC-3'

For the first round of PCR:
A sense internal primer was combined with an antisense external primer and the template DNA used was GFP-PKCzeta CD.
An antisense internal primer was combined with a sense external primer and the template DNA used was GFP-PKN1 CD.

GFP-49aa was generated with the following primers using GFP-PKN1 CD as a template and again incorporated a Xho1 and Apa1 restriction site at the 5' and 3' ends respectively of the PCR product. The resulting PCR product was cloned into pEGFP-C1 to give an N-terminal GFP tag in frame with the PKN1 peptide sequence.
Sense 5'-CCCGCTCGAGCGGCCGCCGCCGCCGGTGGGGACCTGATGCTGCACA TCCAC-3'
Antisense 5'-CGCGATGGGCCCCTAGAGCAAATTGTCCAACTTCAGGTCCCTGT

A-3'

2.6.2 Confocal Microscopy

Cells were seeded on acid washed coverslips and transfected and stimulated as indicated in figure legends. After stimulation cells were washed once with PBS and fixed in 4% (w/v) paraformaldehyde/PBS for 10 mins at RT. When staining for immunoflourescence the following procedure was followed:

1) Aldehyde functions were quenched with 50mM NH_4Cl for 10 mins.
2) Cells were permeabilised and non-specific sites blocked with 0.1% Triton-X-100, 2% BSA (bovine serum albumin) (v/v) in PBS for 15 mins.
3) Incubated in primary antibody for 1 hour. To visualise the actin cytoskeleton phalloidin-Alexa546 (Molecular Probes) was used in place of primary and secondary antibodies. Antibodies/phalloidin-Alexa546 were used at a 1:200 and diluted in PBS containing 2% BSA.
4) Coverslips were washed three times in PBS.
5) Incubated in dye-conjugated secondary antibody for 1 hour shielded from light. Antibodies were used at 1:500 diluted in PBS containing 2% BSA.
6) Coverslips were washed three times in PBS and then washed once in water.
7) Coverslips were mounted on glass slides under MOWIOL (100mM TrisHCl pH 8.8, 10% (w/v) MOWIOL 4-88 (Calbiochem) and 25% (v/v) glycerol) containing anti-photobleaching agent (2.5% (w/v) 1,4-diazabicyclo[2.2.2]octane). Slides were incubated at 37° C for 1 hr to allow MOWIOL to set. Slides were washed and stored at 4°C.

Slides were examined using a confocal laser scanning microscope (Axioplan2 with LSM510 software, Carl Zeiss Inc.) equipped with a 63x/1.4 Plan-APOCHROMAT oil immersion objective. GFP and AlexaFluor-488 (Molecular Probes) were excited with 488nm line of an Argon laser. RFP and Alexa546 were excited with 543nm line of a Helium-neon laser. Individual channels were scanned sequentially to prevent bleed through. Each image represents a single 1μm 'Z' optical section and is representative of at least 10 fields of cells and at least three separate experiments.

2.7 Polyacrylamide gel electrophoresis (SDS-PAGE)

Cell lysate samples were prepared in NuPAGE LDS sample buffer 0.2M DTT, heated at 90°C for 10 mins and briefly sonicated to shear genomic DNA. Samples were run on NuPAGE 4-12% gels with MOPS SDS Running Buffer containing NuPAGE Antioxidant at 200V (constant). RPN800 Full Range Rainbow Markers were run alongside each set of samples.

2.7.1 Western blotting

Proteins separated on NuPAGE gels were transferred electrophoretically onto polyvinylidene difluoride membrane (PVDF). A wet transfer system using Biorad apparatus was used and all transfers were performed at 4°C at constant voltage and 400mA/hour. After transfer membranes were blocked by incubation at RT for 1 hour in 3% (v/v) BSA/TBS-T and then incubated overnight at 4°C in primary antibody diluted 1:1000 in 3% (v/v) BSA/TBS-T. Membranes were washed 3 x 20 minutes in TBS-T followed by incubation with the relevant secondary antibody diluted 1:5000 in 3% (v/v) BSA/TBS-T. Membranes were washed 3 x 20 minutes in TBS-T and developed using enhanced chemiluminesence (ECL) or ECLplus according to the manufacturers guidelines and exposed to Hyperfilm.

2.7.2 Biomolecular modelling

Protein sequences were obtained from NCBI collection, accession numbers; PKCθ NP_006248; PKCζ NP_002735; PKN1 NP_002732. These sequences were aligned in the programme ClustalX to allow the equivalent residues of PKN1aa699-747 to be found in the PKCζ and PKCθ kinase domains. This was then used to highlight the appropriate amino acids in the crystal structure of the PKCθ kinase domain (solved by (Xu et al., 2004)) in the programme SwissModel.

2.7.3 Cell assays and transfection

2.7.3.1 Transfection of plasmid DNA

Transient transfections were performed using Lipofectamine 2000 and following the manufacturers guidelines. DNA/Lipofectamine complexes were prepared in Optimem and added directly to the culture medium. 6 hrs post transfection the media was refreshed and subsequent manipulations were typically performed 48 hrs post-transfection.

2.7.3.2 Transfection of siRNA oligos

HiPerFect reagent was used to achieve knockdown of PKN isoforms using targeted siRNA oligos. Complexes were prepared in DMEM in the absence of serum and oligos targeting PKN isoforms were used at 5nM each. An oligo targeting luciferase was used as a control in all experiments and also to balance the concentrations of oligos used so that in all cases the final concentration of oligo was 15nM. Complexes were added to cells dropwise and incubated under normal growth conditions for 24 hrs after which time the media was replaced. All assays were carried out 48-72 hrs post-transfection.

OLIGOS:

NAME	**TARGET DNA SEQUENCE**
LUC control	AATCGAAGTATTCCGCGTACG
PKN1a	AAGGGCACGGGAACTGGAGTT
PKN1b	AACTGGAGTTGGCTGTGTTCT
PKN2 (PKN2-3)	AAGCATGGCATGTGTCTCTATT
PKN3 (GB3)	GAGAGCCTGTACTGCGAGAAG

2.7.3.3 Hyperosmotic stress

Transiently transfected cells were subjected to hyperosmotic conditions 48 hrs post-transfection for 30 mins by the addition of DMEM containing 0.4M sucrose or 0.5M urea and 25mM Hepes pH7.2. This is equivalent to an increase in osmolarity from 340

mOsmol/kg to 740 mOsmol/kg. After 30 mins the cells were washed quickly with cold PBS and then fixed with 4% (w/v) PFA in PBS. The osmolarity of media was measured using an Osmomat 030 cyoscopic osmometer.

2.7.3.4 Cell fractionation

NIH3T3 cells were transiently transfected (see 2.7.3.1) with plasmids as descibed in figure legends. 48 hours post transfection cell manipulations were performed to examine the cellular distribution of endogenous and over-expressed proteins.

2.7.3.4.1 Cellular distribution and solubility of PKN1

NIH3T3 cells were transfected in 6 well plates and subsequently harvested in 200µl of digitonin buffer (0.05% digitonin, 50mM TrisHCl pH7.5, 150mM NaCl and 1 Complete protease inhibitor tablet per 50ml). Samples were tumbled at 4°C for 30 minutes and then spun at 14000rpm in a bench top centrifuge for 15 minutes. The supernatent was removed and LDS sample buffer added. The pellet was resuspended in 200µl of Triton-X-100 buffer (0.5% T-X-100, 50mM TrisHCl pH7.5, 150mM NaCl and 1 Complete protease inhibitor tablet per 50ml) and the samples were tumbled at 4°C for 30 minutes and then spun at 14000rpm in a bench top centrifuge for 15 minutes. The supernatent was removed and LDS sample buffer was added to both the supernatent and the pellet samples. All samples were analysed by western blot to examine the distribution of the GFP-tagged chimeric proteins (see figure legends).

2.7.3.4.2 Sucrose gradient

15cm dishes of transfected NIH3T3 cells were washed with cold PBS and harvested in 1ml of homogenisation buffer (0.25M sucrose, 10mM Hepes-KOH pH7.2, 1mM EDTA pH7.5, 1mM MgAc and 1 Complete protease inhibitor tablet per 50ml). Samples were homogenised using a cell cracker (EMBL) and spun at 3200rpm for 7 minutes in a bench top centrifuge at 4°C. The pellet was retained for analysis of protein composition by western blot and the supernatent was loaded onto a 12ml sucrose gradient ranging

from 0.3M to 1.2M sucrose and this was spun at 26000 rpm for 18 hours. 1ml fractions from the gradient were then collected and sample buffer added prior to protein distribution being analysed by western blot.

2.7.3.5 Scratch wound assay

Wounds were created in a confluent monolayer of cells by gently scraping the surface with a Gilson D200 pipette tip. After the wound was created the media was replaced with DMEM + 10% FCS, if filming was going to be performed the media also contained 25mM Hepes pH 7.2. For filming scratch wound assays were performed in 24-well plates 72 hrs post-transfection with siRNA oligos and a single vertical wound was made in each well. Plates were sealed around the edge and a small hole made in the side to allow a needle to be inserted through which $C0_2$ was blown. A lowlight microscope with moving stage and heat box was used to follow the wound closure over 24 hrs. A 5x phase objective was used and a picture taken every 15 mins. After the acquisition had finished the cells were harvested by removing the media, washing once with warm PBS and the adding 50µl 2 x LDS Sample buffer to each well and western blots were performed to confirm successful knockdown of PKN isoforms. The pictures were analysed with MetaMorph software to calculate the area of each wound at each time point up to the point of complete closure of the wound. These values were used to give an arbitary speed of migration for each transfection condition and an average of the replicates (at least three) was calculated.

For the multiple scratch wound assays used to analyse the PtdOH content by LC-MS/MS, 15cm confluent dishes of cells were either wounded with 20 vertical and 20 horizontal scratches or left unwounded. In each case the media was replaced at the point of wounding and cells were incubated under normal growth conditions for 4 hrs. After 4 hrs plates were placed on ice, media was removed and cells were washed once with cold PBS. The PBS was removed and the cells were scraped to harvest and snap frozen on dry ice in pre-chilled silanated glassware. Samples were stored at -80°C until lipid extractions were performed (see 2.7.4).

2.7.3.6 Alcohol trap assay

Confluent wells of cells were incubated in either DMEM + 10% FCS containing either 1% EtOH, 0.1% BtOH or 0.1% Bt-2-OH for 1 hr at 37^0C, 5% CO_2. Wounds in the confluent layer of cells were created as before and the media replaced with warm DMEM + 10%FCS containing the appropriate alcohol. Filming of the wounds and measurement of the speed of migration was as described in 2.7.3.5

2.7.3.7 Boyden chamber migration assay

The underside of Transwell inserts (Corning) were coated with either 10μg/ml laminin (from human placenta-Sigma L6274), 25μg/ml fibronectin (from bovine plasma-Sigma F1141) or 50μg/ml collagen (from calf skin-Sigma C8919). These coatings were performed overnight at 4°C and then the membranes were washed and blocked in 0.1% BSA for 1 hr at RT. The membranes were washed again with PBS and placed into fresh wells of a 24 well plate and used immediately.

72 hrs post-transfection with siRNA oligos cells were harvested by trypsin treatment and resuspended in DMEM + 10%FCS. The number of viable cells for each sample was counted using a Beckman Coulter Counter. An equal number of viable cells from each transfection condition were pelleted by centrifugation at 1000g for 5 mins. The cell pellet was gently resuspended in DMEM + 1% FCS to a final concentration of 4 x 10^4 cells/ml. 500μl of DMEM + 10%FCS was placed in the bottom of each well of the plate and 500μl of cell suspension (i.e. 2 x 10^4 cells) was added to the upper chamber of each Transwell insert, another sample of cells were taken to analyse the efficiency of knockdown by western blot.

The plates were incubated at 37^0C, 5% CO_2 for 5 hrs. After 5 hrs cells remaining in the upper chamber of the Transwell insert were removed by gently cleaning the upper surface of the membrane with a damp cotton bud and washing once in PBS. Cells on the lower surface of the Transwell membrane were fixed in 4% PFA. Cells were permeabilised with 0.1% T-X-100 in PBS for 10 mins, stained with DAPI (1:10000 dilution of bisBenzimide H33342 trihydrochloride (Sigma B2261) in PBS) for 5 mins and inserts were washed x 3 with PBS. The extent of cell migration was calculated by

counting of cells on the lower surface of the membrane. This was achieved using a Discovery microscope to take nine pictures of each membrane, these pictures were analysed using Metamorph software and journals to count the number of nuclei in each picture.

2.7.3.8 Boyden chamber invasion assay

0.5ml of warm DMEM was added to each BD BioCoat Growth Factor Reduced MATRIGEL Invasion Chamber and the matrix was allowed to rehydrate for 2 hrs at 37^0C, 5% CO_2. After rehydration 250µl of the DMEM was carefully removed and 750µl of DMEM + 10%FCS was placed in the bottom of each well of the plate. Cells were harvested with trypsin 48 hrs post-transfection with siRNA oligos and resuspended in DMEM + 10%FCS. The number of viable cells for each sample was counted using a Beckman Coulter Counter. An equal number of viable cells from each transfection condition were pelleted by centrifugation at 1000g for 5 mins. The cell pellet was gently resuspended in DMEM + 1% FCS to a final concentration of 4 x 10^4 cells/ml. and 500µl of cell suspension (i.e. 2 x 10^4 cells) were added to the upper chamber of each Boyden chamber, another sample of cells were taken to analyse the efficiency of knockdown by western blot.

The plates were incubated at 37^0C, 5% CO_2 for 24 hrs. After 24 hrs cells and the matrix in the upper chamber of the Boyden chamber were removed by gently cleaning the upper surface with a damp cotton bud. The inserts were processed and the number of cells that had invaded were counted as for the Boyden chamber migration assays (2.7.3.5).

2.7.4 Lipid Extractions

1) All glassware (glass universals, specimen tubes, funnel, chromacol screw-top vial and Pasteur pipettes) was silanated prior to use. Glassware was soaked in a 3% solution of dimethyldichlorosilane in HPLC grade toluene in a fume hood for

1 hr, rinsed twice with methanol and twice with HPLC grade water and allowed to dry.

2) Two mixures of internal standards are prepared: one containing the PIs (IS) (DPPI(4)P, DPPI(4,5)P_2 and DPPI(3,4,5)P_3) at 100μg/ml in chloroform:methanol:water (C:M:W) at 5:5:1 and the other containing other phospholipids (IS2) (DLPC, DLPA, DLPS, DLPG, DLPE and DPPI) at 100μg/ml in C:M:W at 5:5:1
3) 200μl of sample is added to 4ml of acidified C:M (2.5:1) in a glass universal.
4) The mixture is probe sonicated for 10 secs at power 22 and left at RT for 1 hr.
5) Samples are filtered through a 0.22μm membrane resistant to chloroform by vacuum filtration and collected in a specimen tube.
6) The sample is supplemented with 0.2 volumes of K_4EDTA, transferred to a chromacol screw-top vial using a Pasteur pipette and spun at 800g for 15 mins at 4°C.
7) The organic phase is collected, transferred to a fresh chromacol tube and dried at 37 °C under nitrogen using a sample concentrator.
8) The lipid pellet is resuspended in 100 μl C:M:W (5:5:1) and transferred to a 100μl silanised insert using a 100μl gastight syringe. The insert is placed in an amber vial and the lipids dried under nitrogen.
9) 2μg of IS and 2μg of IS2 are added and dried under nitrogen.
10) Samples are stored art -80°C until analysed on an HPLC-tanderm mass spectrometer by members of the Biophysics laboratory at CR-UK.

Chapter 3

PKN1 and hyperosmotic stress

3 Introduction

As was discussed in Chapter 1 the temporal and spatial control of kinase activity is vitally important for appropriate responses to be mounted to specific stimuli. PKN1 has been demonstrated to have multiple roles in signal transduction (see 1.8.1) and so control over the localisation of PKN1 activity is likely to be important.

An examination of PKN1 localisation in response to various stimuli had previously uncovered a role for PKN1 as a stress responsive kinase. It was demonstrated that in response to hyperosmotic stress ectopically expressed PKN1 translocated in a reversible manner to punctate structures (Torbett et al., 2003). Initially it was thought likely that the structures observed in response to hyperosmotic conditions were membrane bound vesicles particularly as it has been shown previously that Rho B can target PKN1 to endosomes (Mellor et al., 1998) and regulate traffic of the epidermal growth factor receptor (Gampel et al., 1999). However no evidence of the hyperosmotic induced structures co-staining with common endosomal markers or markers of other vesicular structures such as the Rab proteins was found. The nature of the structures that PKN1 is targeted to remains unknown but it is known that the recruitment of PKN1 is dependent on Rac1. Interestingly one of the consequences of hyperosmotic stress is a remodelling of the actin cytoskeleton (see 1.8.1) and Rac1 is an important regulator of the cytoskeleton (see 1.6.1). It is therefore possible that PKN1 is involved in this reorganisation of the cytoskeleton.

This chapter seeks to address how PKN1 is targeted to these structures in response to hyperosmotic stress in an attempt to further our understanding of the role of PKN1 in this hyperosmotic stress response

3.1 PKN1 translocation is triggered by mechanical stress

The translocation of PKN1 was described previously to occur in response to hyperosmotic stress caused by exposure to 0.4M sucrose. Sucrose is membrane impermeant and so causes an external increase in osmolarity resulting in an efflux of water from the cell. This results in an increase in intracellular ionic strength and causes cells to shrink so the hyperosmotic stress is accompanied by a mechanical stress component. Therefore to address the question of whether the PKN1 translocation is induced by intracellular hyperosmolarity or mechanical stress the effect of sucrose vs urea induced hyperosmotic stress was examined. Urea is membrane permeant and so hyperosmotic stress induced by it results in both an internal and external increase in osmolarity and no overall efflux of water from the cell and hence no mechanical stress. GFP-PKN1 was expressed in NIH3T3 cells and the effect of an increase in osmolarity to 740 mOsmol/Kg for 30 minutes was examined. As previously observed exposure to a hyperosmotic stress caused by sucrose resulted in an accumulation of GFP-PKN1 in large punctate structures. However a similar increase in osmolarity caused by exposure to urea did not result in any gross change in GFP-PKN1 localisation (Figure 3:1). This suggests that the PKN1 translocation is induced by the mechanical stress of an increase in osmolarity caused by sucrose rather than the concurrent increase in intracellular ion concentration.

GFP-PKN1 overexpression is also seen to affect the actin cytoskeleton under hyperosmotic conditions. In response to hyperosmolarity caused by both sucrose and urea actin is redistributed from filaments to a cortical ring around the edge of the cell. However in GFP-PKN1 expressing cells in response sucrose treatment actin colocalises with PKN1 in punctate structures. In response to urea treatment however GFP-PKN1 expression instead stabilises actin in filaments.

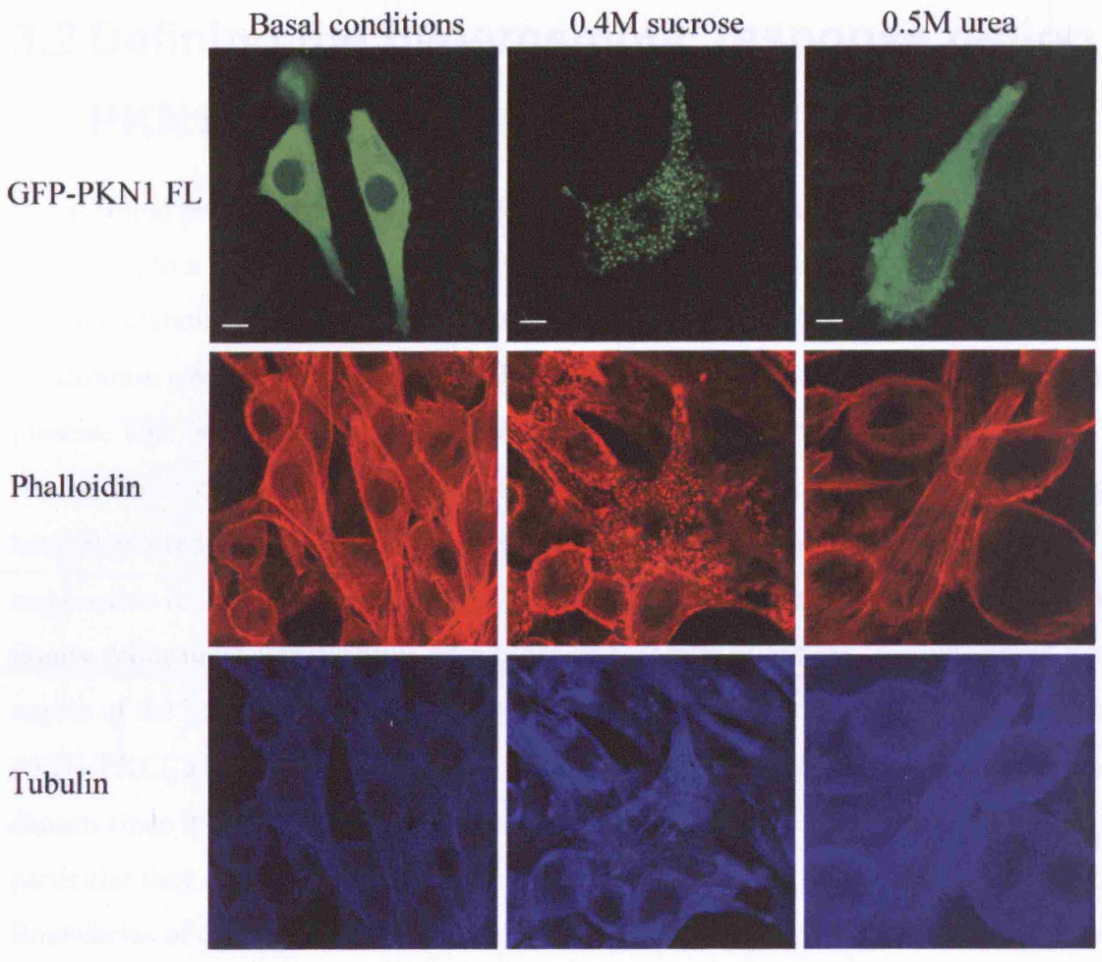


Figure 3:1 GFP-PKN1 translocates in response to mechanical stress and affects the rearrangement of the actin cytoskeleton under conditions of hyperosmolarity.

NIH3T3 cells were transiently transfected with GFP-PKN1 and either untreated (basal conditions) or subjected to 30 minutes of hyperosmolarity by treatment with either 0.4M sucrose or 0.5M urea. After fixation actin was visualised using phalloidin-Alexa546 and stained for tubulin expression. All images are a single 1.0μM 'Z' section and are representative of at least 30 fields from three independent experiments, the scale bar is equivalent to 10μM.

3.2 Defining the hyperosmotic response region of PKN1

It was found previously that both PKN1 and PKN2 show similar changes in localisation in reponse to a transient increase in osmolarity to 740 mOsmol/Kg (Dr Torbett, personal communication). It was known that the kinase domain of PKN1 controlled this localisation since the kinase domain alone displays the same response as the full-length protein. This behaviour is specific to the PKN isoforms compared to other closely related kinase domains such as those of PKCζ and PKCε. This is intriguing as it might have been predicted that the regulatory domain of the PKN isoforms would be responsible for the dynamic regulation of their localisation since this is where the PKN family differ most significantly from other PKC family members. To understand which region of the kinase domain conferred the PKN specific behaviour, the localisation of PKN1/PKCζ kinase domain chimeras were examined. The PKCζ kinase domain was chosen since it has a high degree of homology to those of the PKN isoforms and in particular they all contain acidic residues in the hydrophobic "phosphorylation" site. Boundaries of the chimeras were decided based on molecular modelling and sequence homology of the two kinase domains to minimise the chance of the chimeric proteins being misfolded (Figure 3:2). All the chimeras were N-terminally GFP tagged and transiently over-expressed in NIH3T3 cells. Their localisation in response to 30 minutes of hyperosmolarity caused by 0.4M sucrose was examined and compared to the PKN1 and PKCζ kinase domains.

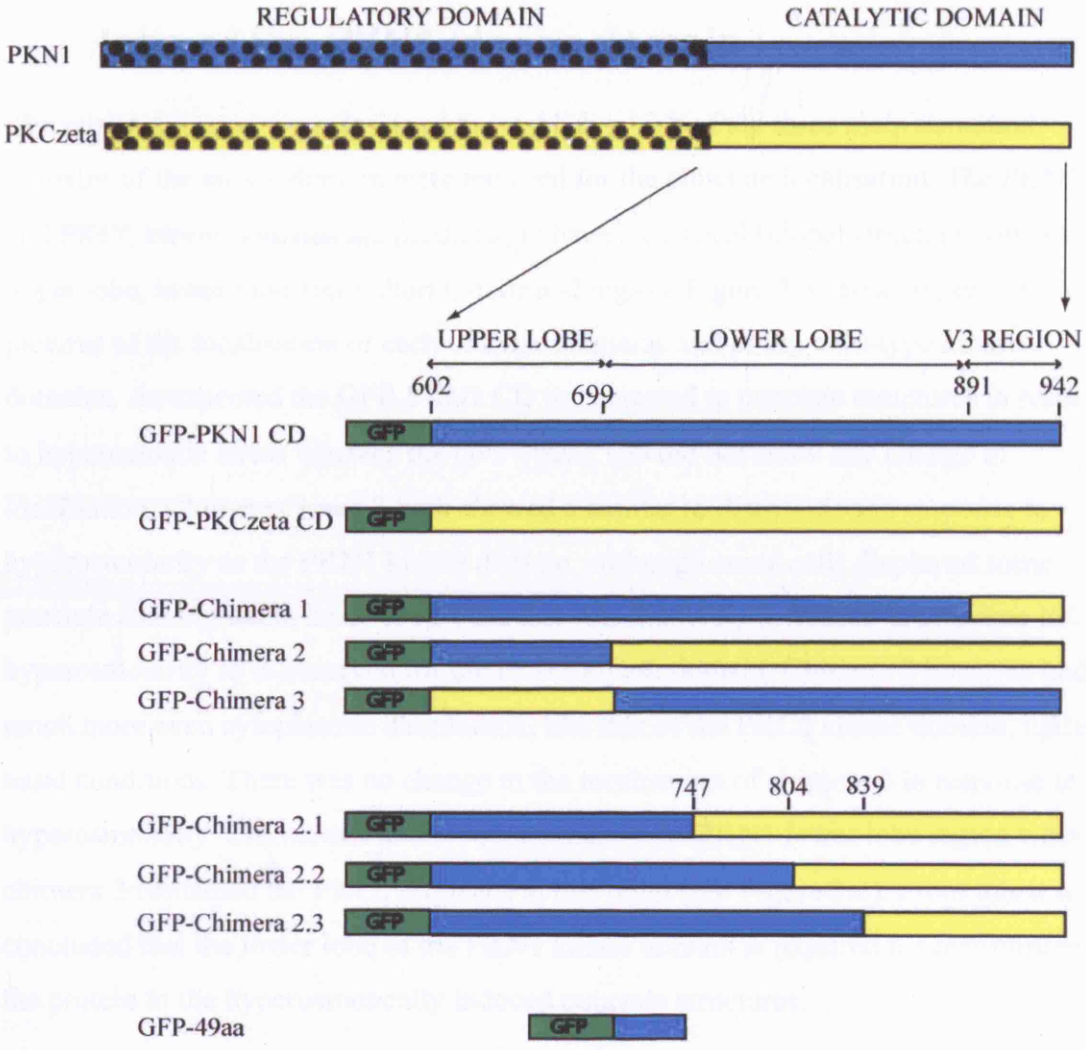


Figure 3:2 Design of PKN1/PKCzeta chimeras.

Chimera boundaries were designed based on sequence comparisons of the PKN1 and PKCζ kinase domains to limit the possibility of the chimeras being misfolded. They were cloned into pEGFP-C1 to enable expression in frame with a N-terminal GFP tag (see 2.6.1.8 for cloning strategy).

3.2.1 The osmotic response element is within the lower lobe of the PKN1 kinase domain

The initial chimeras were designed to establish which of the three main structural domains of the kinase domain were required for the punctate localisation. The PKN1 and PKCζ kinase domains are predicted to have a classical bilobal structure with an upper lobe, lower lobe and a short C-terminal region. Figure 3:3 shows representative pictures of the localisation of each of these chimeras and of the wild-type kinase domains. As expected the GFP-PKN1 CD translocated to punctate structures in response to hyperosmotic stress whereas the GFP-PKCζ CD did not show any change in localisation. Chimeras 1 and 3 both showed a similar re-distribution in response to hyperosmolarity as the PKN1 kinase domain. Although some cells displayed some punctate staining under basal conditions this was markedly increased in response to hyperosmolarity as is observed for the PKN1 kinase domain. Chimera 2 however had a much more even cytoplasmic distribution, like that of the PKCζ kinase domain, under basal conditions. There was no change in the localisation of chimera 2 in response to hyperosmolarity. Chimeras 1 and 3 both contained the PKN1 lower lobe region wheras chimera 2 contained the PKCζ sequence at this point (see Figure 3:2). From this it was concluded that the lower lobe of the PKN1 kinase domain is required for recruitment of the protein to the hyperosmotically induced punctate structures.

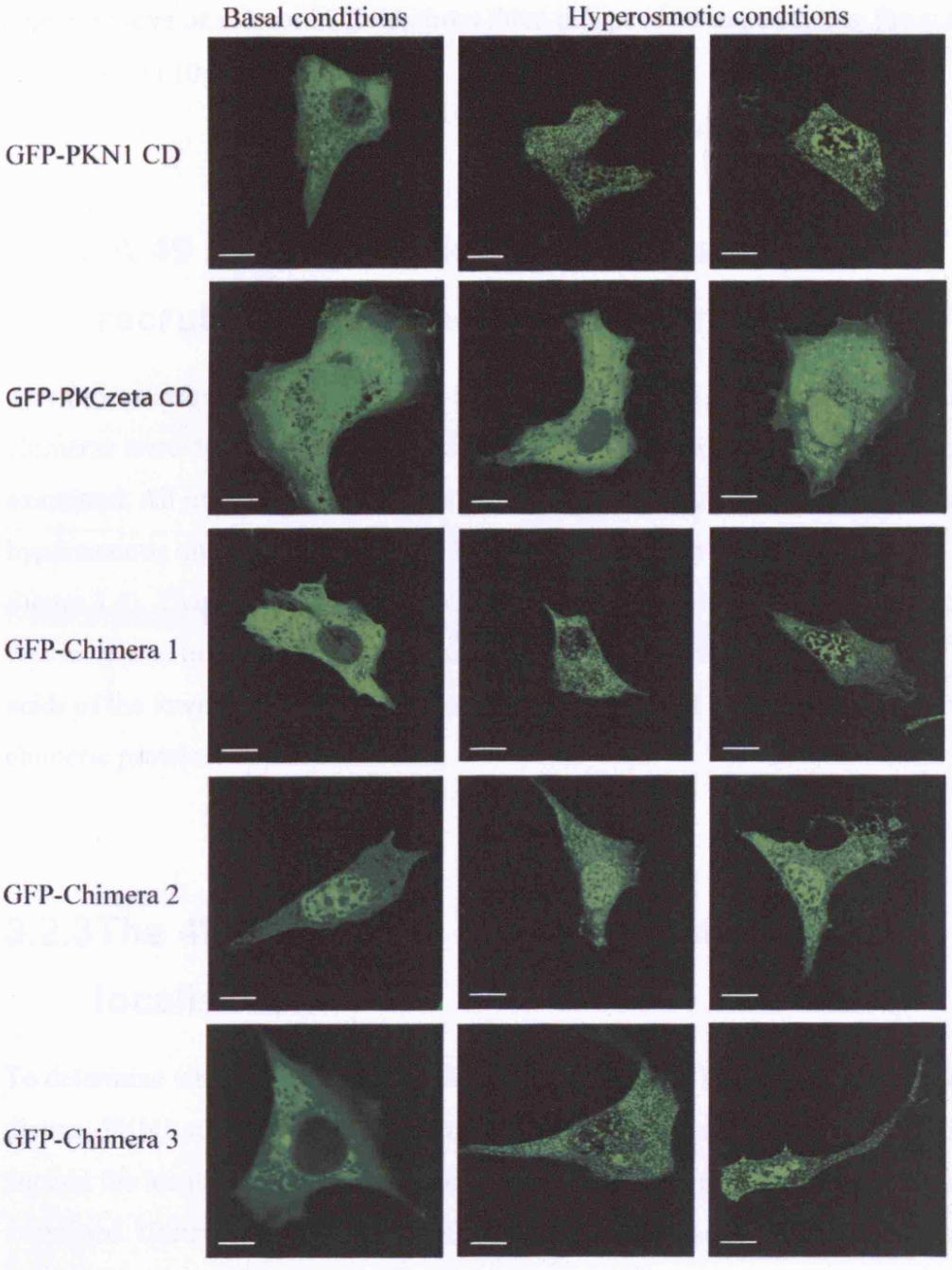


Figure 3:3 The lower lobe of the PKN1 kinase domain is required for translocation of the protein in response to hyperosmotic stress.

NIH3T3 cells were transiently transfected with GFP-PKN1 CD, GFP-PKCzeta CD or GFP-Chimera 1/2/3 and either untreated (basal conditions) or subjected to 30 minutes of hyperosmolarity by treatment with 0.4M sucrose (hyperosmotic conditions). The scale bar is equivalent to 10μM. All images are a single 1.0μM 'Z' section and are

representative of at least 30 fields from three independent experiments, the scale bar is equivalent to 10μM.

3.2.2 A 49 amino acid domain is necessary for recruitment to punctate structures

To further narrow down the osmotic responsive region of PKN1 another series of chimeras were designed and their localisation in response to hyperosmotic stress examined. All of these new chimeras behaved in the same way both under basal and hyperosmotic conditions as the PKN1 kinase domain (see Figure 3:4). That is they all displayed some punctate staining under basal conditions that was increased in response to hyperosmolarity. This suggests that the initial 49 amino acids of the lower lobe region are necessary for the PKN1-like localisation of the chimeric proteins.

3.2.3The 49 amino acid domain is sufficient for iocaiisation

To determine whether the 49 amino acids were sufficient for localisation to these distinct PKN1 structures these 49 amino acids were expressed with an N-terminal GFP tag and the localisation of this peptide under basal and hyperosmotic conditions examined. Under basal conditions this peptide was localised primarily in punctate structures and no change in the number or size of vesicles was seen in response to hyperosmolarity. To confirm that the localisation of the GFP-49aa peptide was the same as that of the full-length protein, DsRed-PKN1 FL was co-expressed with the GFP-49aa construct in NIH3T3 cells. Consistent with the behaviour of GFP-PKN1 FL the DsRed-PKN1 FL had a diffuse cytoplasmic distribution under basal conditions but translocated to punctate structures in response to hyperosmolarity. Under hyperosmotic conditions in cells co-expressing both DsRed-PKN1 FL and the GFP-49aa both proteins are seen to

localise to punctate structures and a large number of these structures are positive for both proteins (Figure 3:5).

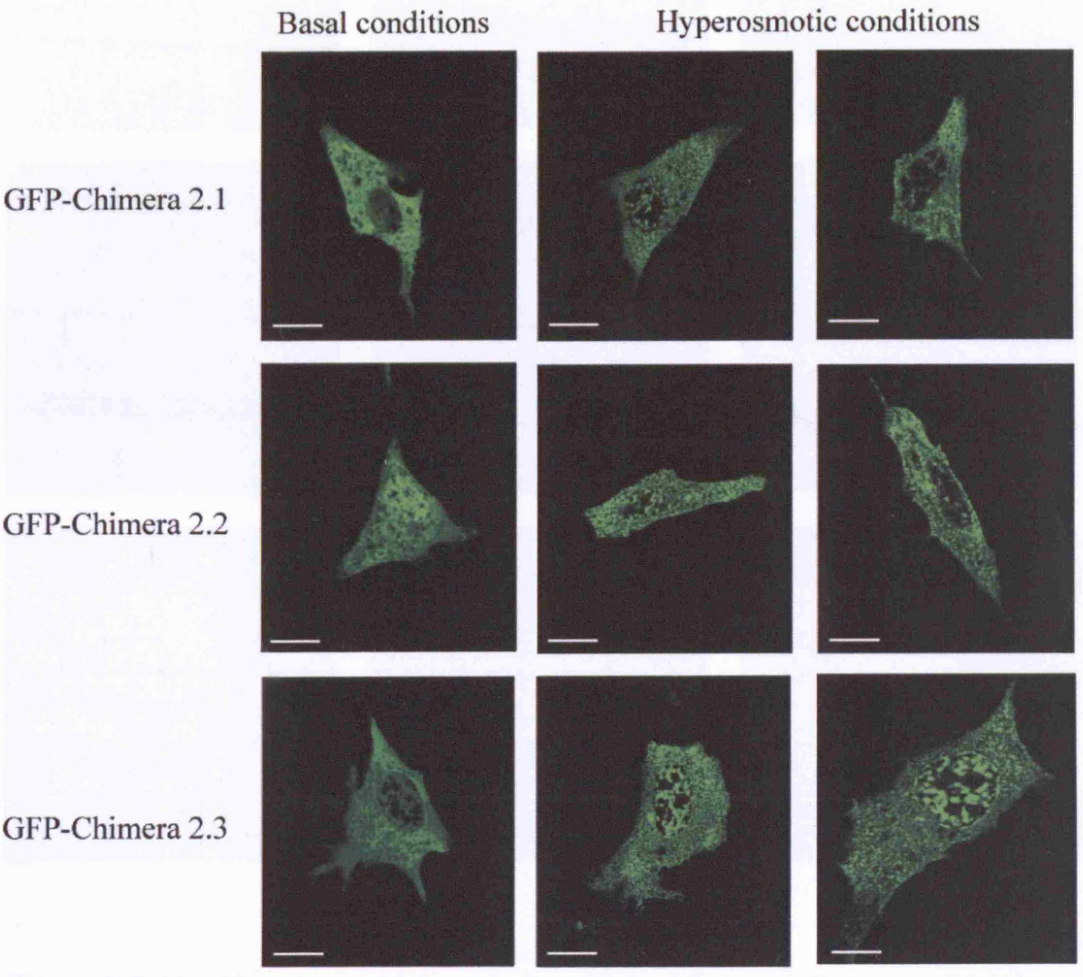


Figure 3:4 The initial 49 amino acids of the lower lobe are required for hyperosmotic induced recruitment to punctate structures.

NIH3T3 cells were transiently transfected with GFP-Chimera 2.1, 2.2 or 2.3 and either untreated (basal conditions) or subjected to 30 minutes of hyperosmolarity by treatment with 0.4M sucrose (hyperosmotic conditions). All images are a single 1.0μM 'Z' section and are representative of at least 30 fields from three independent experiments, the scale bar is equivalent to 10μM.

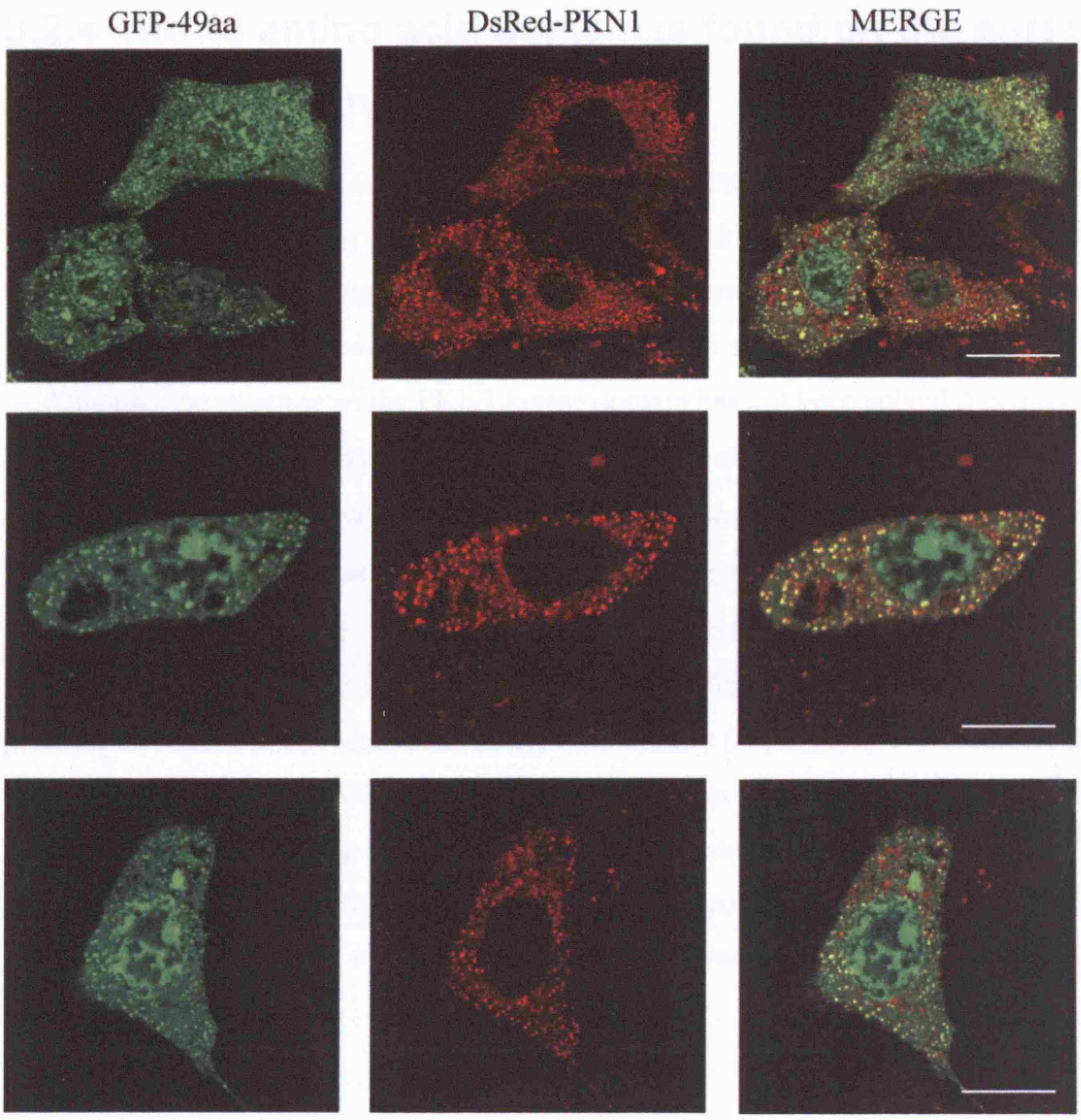


Figure 3:5 The 49 amino acid domain is sufficient for recruitment to PKN1 hyperosmotic induced structures.

NIH3T3 cells were transiently co-transfected with DsRed-PKN1 and GFP-49aa and subjected to 30 minutes of hyperosmolarity by treatment with 0.4M sucrose. All images are a single 1.0μM 'Z' section and are representative of at least 30 fields from three independent experiments, the scale bar is equivalent to 10μM.

3.2.4 The 49 amino acid domain is found on the surface of the protein.

The 49 amino acid domain found to be responsible for PKN1 recruitment to punctate structures in response to hyperosmolarity consists of residues 699-747 found at the start of the lower lobe of the kinase domain. It would be informative to know whether the domain is exposed or buried within the protein structure and what its tertiary structure is. Although the structure of the PKN1 kinase domain has not been solved it is possible to make some predictions based on the solved structure of the PKCθ kinase domain (Xu et al., 2004) since the primary sequences of the PKN1 and PKCθ kinase domains are highly similar. ClustalX was used to align the sequences of the PKN1, PKCζ and PKCθ kinase domains (see Figure 3:6). This alignment was then used to highlight in the PKCθ structure the location of the PKN1 domain responsible for the recruitment of PKN1 to punctate structures under conditions of hyperosmolarity (see Figure 3:7). It can be seen from this modelling that the novel 49 amino acid domain is found on the surface of the protein and has a helix-loop-helix structure. This suggests that this domain would be accessible to interact with lipid or protein partners that could recruit the protein to a distinct cellular location in response to hyperosmotic stress.

Figure 3:6 ClustalX alignment of PKN1 PKCζ and PKCθ kinase domains.

The 49 amino acid domain identified to be sufficient for recruitment to punctate structures is shown bounded by the red box.

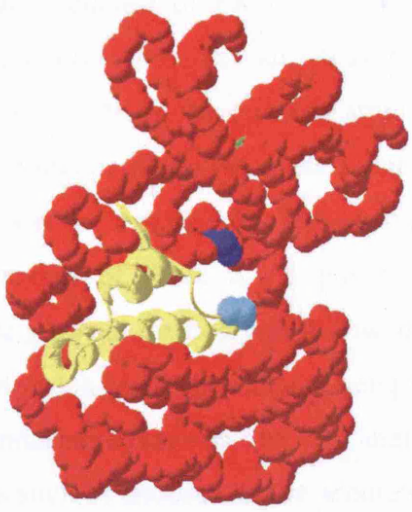


Figure 3:7 Location and structure of the 49 amino acid domain.

The programme ClustalX was used to produce an alignment between the sequences of the kinase domains of PKN1 and PKCθ. This alignment allowed the relevant 49 amino acids in the PKCθ structure to be highlighted in yellow. Critical catlytic residues are also highlighted to demonstrate the position of the active site, VAIK-green, DFG-dark blue, HRD-turquoise (see Table 2 in Section 1.3.1).

3.3 Cellular distribution of PKN1

Having identified a discrete sequence of PKN1 that is responsible for targeting the protein to the punctate structures the next aim was to identify the nature of the interaction that occurs to target PKN1 to the punctate structures. Purifying the structures and carrying out a proteome and/or lipidome of the complex would result in a greater understanding of the hyperosmotic response signalling pathway PKN1 lies on and guide further studies on the function of these novel punctate structures. To purify the structures a classical sucrose gradient method was followed designed for the purification of small membrane structures such as endosomes to attempt to obtain a fraction enriched in the hyperosmotically induced structures. In this method protein complexes and membrane-bound structures such as endosomes are separated from cytoplasmic proteins based on their density such that free cytoplasmic proteins are found in low density fractions and structures such as endosomes are found in higher density fractions. NIH3T3 cells were used to examine the distribution of endogenous PKN1, GFP-49aa (which is constituitively localised to punctate structures) or GFP-Chimera 2 (which is not localised to hyperosmotically induced structures) under either basal or hyperosmotic conditions (see Figure 3:8). From these results it can be seen that endogenous PKN1 is found primarily in the lower density fractions (2 and 3) both under basal and hyperosmotic conditions with a slight enrichment in higher density fractions under hyperosmotic conditions. The distribution of GFP-Chimera 2 is similar to that of the endogenous PKN1 with it being found predominantly in the lower density fractions. Most interestingly although the soluble GFP-49aa is distributed throughout the fractions the majority of this protein is found within the pellet material that is cleared from the cell lysate prior to the lysate being loaded onto the sucrose gradient. Since the GFP-49aa is known be located in the punctate structures (see Figure 3:5) this observation suggests that the PKN1 structures seen to accumulate after hyperosmotic shock are not free in the cytoplasm are therefore not loaded onto the sucrose gradient. The pellet from the lysate clearing centrifugation step contains large organelles such as the nucleus and cellular components linked to the cytoskeleton. It is possible that the GFP-49aa material found in the pellet is nuclear material since some nuclear staining is observed by immunofluorescence. However the GFP-Chimera 2 shows a similar degree of nuclear

localisation as the GFP-49aa but a much lower proportion of the GFP-Chimera 2 is found within the pellet material. This suggests that the punctate structures we were trying to purify by this method are found within the pellet material due to being linked to the cytoskeleton. This is supported by the previous observation that actin colocalises with GFP-PKN1 in the punctate structures in response to hyperosmolarity (see Figure 3:1).

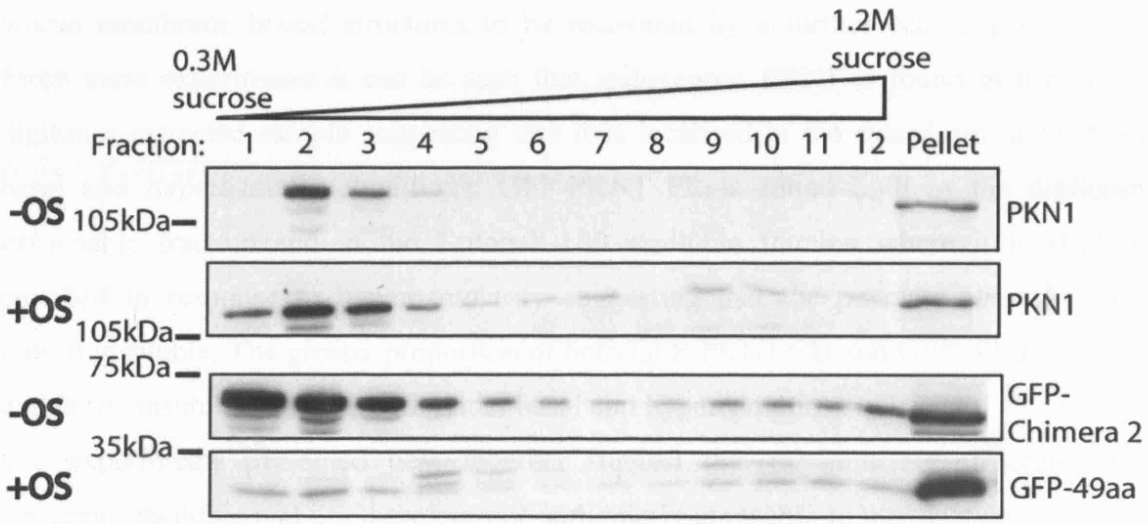


Figure 3:8 Distribution of PKN1 across sucrose gradient fractions.

NIH3T3 cells were transiently tranfected with GFP-Chimera 2 or GFP-49aa; 48 hours post-transfection cells were placed in iso-osmotic or hyperosmotic media for 30 minutes. After 30 minutes treatment cell lysates were prepared by breaking open the cells in a cell cracker. A low speed centrifugation was performed to clear the lysate of cellular debris such as unbroken cells, nuclei and the cytoskeleton and the supernatents from this spin step were loaded onto a continuous sucrose gradient (0.3M to 1.2M sucrose). After overnight centifugation 1 ml fractions were collected and samples of each were run on an SDS-PAGE gel and western blots were carried out to look at the distribution of the transfected GFP-49aa, GFP-Chimera 2 and endogenous PKN1.

To investigate further the cellular localisation of the hyperosmotic induced PKN1 structures cell fractionation procedures were carried out. These involved looking at the solubility of PKN1 in various detergents, the results of such an experiment are shown in Figure 3:9. NIH3T3 cells expressing either GFP-PKN1, GFP-PKN1 CD or GFP-49aa were harvested into a buffer containing 0.05% digitonin to allow cytoplasmic proteins to leak out of the cells and be recovered by centrifugation. The pellet from the centrifugation step was then resuspended in a buffer containing 0.5% Triton-X-100 to solubilise membranes and allow integral membrane proteins and proteins contained within membrane bound structures to be recovered by a further centrifugation step. From these experiments it can be seen that endogenous PKN1 is found in the initial digitonin extracted sample suggesting that it is localised in the cytoplasm under both basal and hyperosmotic conditions. GFP-PKN1 FL is found both in the digitonin extractable fraction and in the Triton-X-100 insoluble fraction where it is slightly enriched in response to hyperosmolarity suggesting that the punctate structures are indeed insoluble. The greater proportion of both GFP-PKN1 CD and GFP-49aa is found within the insoluble fraction both under basal and hyperosmotic conditions.

The experiments presented here together suggest that the punctate structures are detergent insoluble and are therefore not particularly amenable to purification to enable identification of interacting partners.

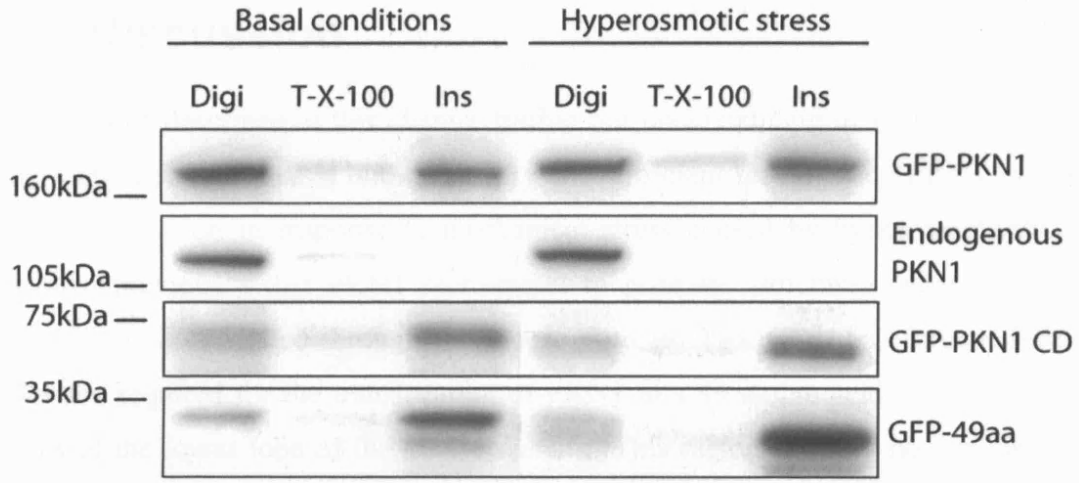


Figure 3:9 Solubility of endogenous and overexpressed PKN1

NIH3T3 cells were harvested into lysis buffer containing 0.05% digitonin 48 hours post-transfection and incubated for 30 minutes at 4°C. Samples were centrifuged at 14000rpm for 15 minutes and LDS sample buffer added to the supernatents (Digi. samples). The pellets from the centrifugation step were resuspended in lysis buffer containing 0.5% Triton-X-100 and incubated for 30 minutes at 4°C prior to a second 14000rpm centrifugation being performed. LDS sample buffer was added to the supernatents (T-X-100 samples) and pellets (Ins. samples) from this second centrifugation. All samples were run on SDS-PAGE gels and distribution of PKN1 and GFP tagged proteins analysed by western blot. The images shown are representative of the results obtained from two independent experiments.

3.4 Discussion

The results described in this chapter further our understanding of PKN1 localisation by defining a domain that is both necessary and sufficient for targeting PKN1 to a distinct cellular location in response to mechanical stress caused by hyperosmolarity. It was known previously that PKN1 recruitment to punctate structures under hyperosmotic conditions is mediated through the kinase domain. This work has further defined the domain required for the translocation of PKN1 to a 49 amino acid stretch found at the start of the lower lobe of the kinase domain. This region displays no similarity to any other known interaction domain and so it remains unclear whether this novel domain interacts with a protein or lipid partner to mediate translocation of PKN1 in response to hyperosmolarity. The constitutive localisation of the tagged 49 amino acid peptide to punctate structures is not unexpected. The localisation of the full length protein was shown previously to be dependent on Rac activation (Torbett et al., 2003). It has been suggested that the interaction of Rho GTPases with the amino terminal HR1 motif of the regulatory region disrupts an autoinhibitory intramolecular interaction and hence causes a change in conformation that allows PKN1 to become activated (Kitagawa et al., 1996). It is therefore logical to propose that Rac binding to the full-length protein in response to hyperosmolarity results in an open conformation of PKN1 which exposes the 49 amino acids at the entrance to the substrate binding pocket. With this domain exposed and available to bind the relevant protein or lipid partner PKN1 is recruited to the punctate structures. However if the domain is expressed alone then it is permanently recruited to the structures by its interaction partner. Unfortunately attempts to identify proteins that interact with this 49 amino acid domain were hampered by the detergent insolubility of overexpressed PKN1, prehaps due to an association with the cytoskeleton. In addition to the work shown here further evidence that the PKN1 complex is insoluble comes from observations made by Dr Adele Casimassima, a post doc in the lab. Dr Casimassima has been working on the PKN1 osmotic response pathway and attempting to reconstitute the signalling complex by co-expressing PKN1, JIP and MKK4 which are all seen by immunofluorescence to colocalise in the vesicular structures after hyperosmotic shock. This work has involved overexpressing these proteins in cells and looking at interactions between them by immunoprecipitation (IP). Routinely, cell lysates are cleared by

carrying out a low speed centrifugation prior to IP and the IP carried out on the supernatent of this centrifugation. In cells subjected to hyperosmotic shock the over-expressed proteins are unable to be immunoprecipitated from the cleared cell lysate. Instead they appear in the pellet from the low speed centrifugation suggesting that the proteins become insoluble due to the hyperosmotic shock.

This chapter also looked at whether PKN1 is recruited to the structures in response to the increase in intracellular ionic strength or by the mechanical stress resulting from hyperosmolarity caused by sucrose exposure. Using urea to cause hyperosmolarity allowed us to address this question and it was found that the same increase in osmolarity did not elicit the same PKN1 translocation. This result implies that the PKN1 translocation is stimulated by the mechanical stress that is caused due to cell shrinkage specifically brought about by cell membrane impermeant solutes such as sucrose. Under hyperosmotic conditions PKN1 was seen to colocalise with actin and the level of disruption of the actin cytoskeleton in response to sucrose treatment was greater in cells overexpressing GFP-PKN1. These results together provide some evidence of PKN1 involvement in the rearrangement of the cytoskeleton and mechanotransduction of signals in response to hyperosmolarity. Consistent with this idea it has been shown by Dr adele casamassima (a post-doc in the lab), that upon hyperosmotic shock proteins of the JNK signalling pathway are co-recruited into this compartment. These include JNK interacting protein (JIP1) and MKK4. Additionally it has been observed that in PKN1 KO MEFs the activation of JNK in response to hyperosmotic shock is impaired. These observations raise the possibility that the PKN1 structures induced by hyperosmotic conditions form a signalling complex somehow linked to the cytoskeleton that is important for cellular changes in response to hyperosmolarity.

The work in this chapter relied on the overexpression of PKN1 to enable a chimera based approach to define the hyperosmotic response region. However it was also found that overexpressing PKN1 affects the cytoskeletal rearrangements in response to hyperosmolarity. This observation coupled with the numerous reports of PKN1 interacting with cytoskeletal components led to an interest in examining the role of endogenous PKN1 in control of the cytoskeleton. An important physiological role for

the cytoskeleton is in controlling cell movement and pathologically cell movement is extremely important in the metastasis of cancer cells. The next chapter will therefore investigate if PKN1 is involved in controlling the migration or invasion of cancer cells.

Chapter 4

PKN1 in migration and invasion

4 Introduction

As was discussed in the introductory chapter cell motility is a multi-step process and much work is going into dissecting the roles different proteins play in the signalling pathways involved. One of the essential processes of cell motility is remodelling of the cytoskeleton to produce the force necessary to drive cells forward. Major regulators of the cytoskeleton dynamics seen in migrating and invading cells are the Rho GTPases (for review see (Raftopoulou and Hall, 2004)). Since the PKNs are downstream effectors of these molecular switches it is of course interesting to speculate on a role for them in controlling cell motility. Additionally the PKNs have, as mentioned before, been reported to have numerous interactions with cytoskeletal components and in the previous chapter overexpression of PKN1 was shown to affect the rearrangement of the actin cytoskeleton. In searching for a role for endogenous PKN1 it therefore seemed appropriate to investigate motility of transformed cells.

Interestingly PKN isoforms have already been shown to have a function in prostate cancer. PKN3 has been found to be an effector of the PI3K pathway in PC3 cells (a prostate cancer cell line) and is required to mediate the invasive cell growth resulting from activation of this pathway (Leenders et al., 2004). PKN1 has been shown to activate the androgen receptor, a ligand-activated transcription factor that is thought to play an important role in the development of prostate cancer (Metzger et al., 2003). The study by Leenders et al is particularly interesting since it implicates PKN3 in control of metastasis of PC3 cells *in vivo,* suggesting that PKN3 regulates cell adhesion or motility.

The results in this chapter describe use of siRNA mediated depletion of the PKN isoforms and inhibition of PKN activity to investigate if the PKNs have a role to play in regulating the migration and invasion of transformed breast cells.

4.1 PKN isoform expression varies between cancer cell lines

To investigate PKN involvement in transformed cell motility it was decided to use cultured transformed cells and *in vitro* motility assays. Transformed cell lines generated from patient tumour samples provide a valuable way of investigating the signalling pathways involved in many different aspects of cancer cell biology. To decide which transformed cell models to use the levels of PKN expression in seventeen different cell lines derived from six different tumour types was examined.

The expression levels of the three PKN isoforms in these breast, lung, prostate, colon, liver and kidney, transformed cells are shown in Figure 4:1. All but one of the cells lines expressed at least one PKN isoform, the exception being the colon HT29 cell line. Most of the cell lines tested expressed all three isoforms to a greater or lesser extent and it does not appear that any one isoform is more likely than the others to be expressed. However the absolute expression levels of each of the three isoforms is not known since the efficiency of detection of the three antibodies may be very different. Comparisons of single isoform expression levels between the different cell lines can be made though. PKN2 expression is lower in the colon, liver and kidney cell lines than in the breast, lung and prostate cell lines. There is no obvious correlation between PKN1 or PKN3 expression levels and cell type.

As discussed previously PKN3 has been implicated in the motility of transformed prostate PC3 cells (Leenders et al., 2004). Prostate cells share similarities to breast cells in that they are both constituents of hormone responsive organs and tumours arising from these tissues are often regulated by hormones (Lange et al., 2007). Therefore it was decided to investigate PKN function in the motility of breast cells. Two of the transformed breast cell lines, the MDAMB-231 and MDAMB-468, display high levels of expression of all three PKN isoforms. However the other transformed breast cells, the MCF7s, predominantly express PKN1. Therefore to examine the role of PKN1 in the motility of transformed cells the MCF cell line was chosen for study and to assess the contribution of all three PKN isoforms to transformed cell motility the MDAMB-468 cells were chosen.

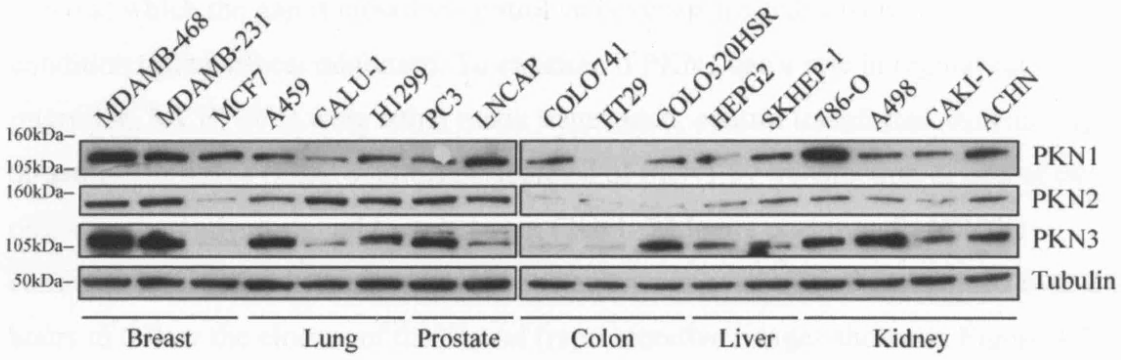


Figure 4:1 PKN expression levels in cancer cell lines.

Cell lysates were prepared from each cell line and 30μg total protein was loaded for each cell line. PKN expression levels were detected with the relevant antibodies. Tubulin levels were detected to demonstrate equal loading of each lysate.

4.2 PKN involvement in migration is cell type dependent.

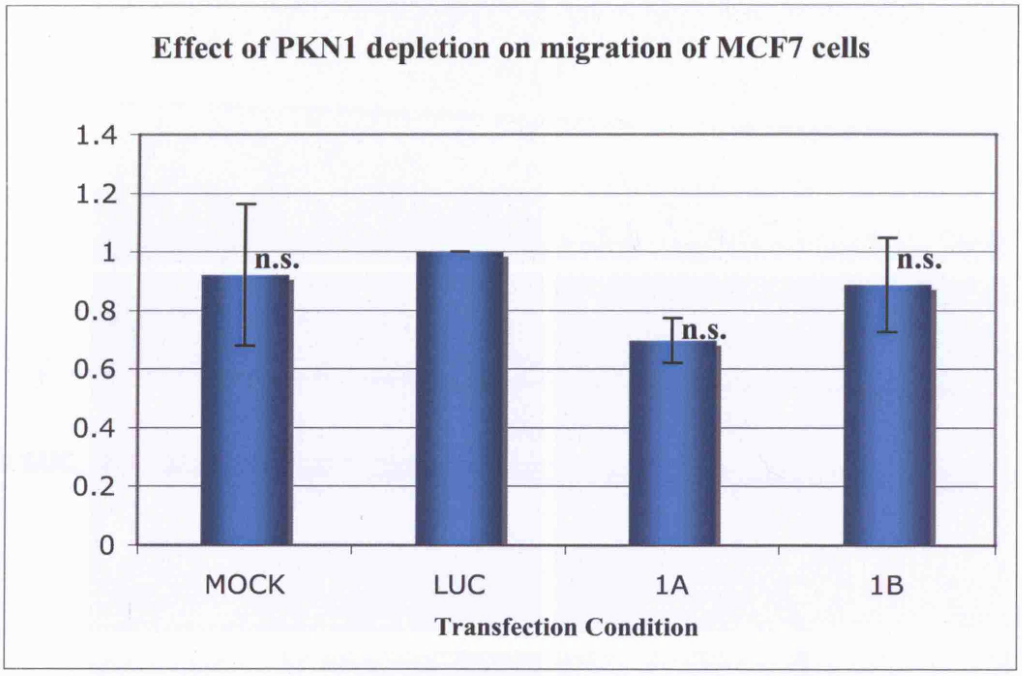
To determine if the PKNs play a role in the motility of two transformed breast cell lines scratch wound assays were employed. This assay measures the speed at which cells migrate to close a gap created in an otherwise confluent layer of cells. By comparing the speed at which the gap is closed in control cells versus treated cells it can be seen if the condition tested affects migration. To examine if PKN1 has a role in regulating migration, MCF7 cells were either mock transfected, control transfected with an oligo targeting luciferase (LUC control) or depleted of PKN1 by transfection of one of two oligos specifically designed to only target PKN1. 72 hours post transfection the confluent layers of cells were wounded and an area of each wound was filmed over 24 hours to follow the closure of the wound (representative images shown in Figure 4:2c). The speed of closure was compared in each case to the LUC control wounds to give the relative speed of migration Figure 4:2a shows the average results from three such experiments. It can be seen that the control LUC transfection had no effect on the speed of migration compared to the MOCK transfection control. The two oligos targeted against PKN1 (1A and 1B) both deplete the levels of PKN1 by about 70% compared to the control transfections (Figure 4:2b). However despite achieving a good level of knockdown of PKN1 no effect on migration is seen. Whilst the average speed of migration relative to the LUC control is decreased upon depletion of PKN1 with both the 1A and 1B oligos this decrease is not statistically significant due to variability in the assay. PKN1 therefore was concluded not to play a rate limiting role in the migration of MCF7 cells in this system.

Unlike the MCF7 cells the MDAMB-468 cells express all three PKN isoforms (see Figure 4:1). Since both PKN2 and PKN3 have been reported to play roles in control of cell motility it was decided to use siRNA to deplete all three isoforms to overcome possible problems of redundancy. Once again a scratch wound assay was used to assess the contribution of the PKN isoforms on migration of this cell line. As can be seen from Figure 4:3b the levels of PKN1 and PKN2 were successfully depleted compared to the LUC control. Unfortunately despite previous successful detection of PKN3 levels using

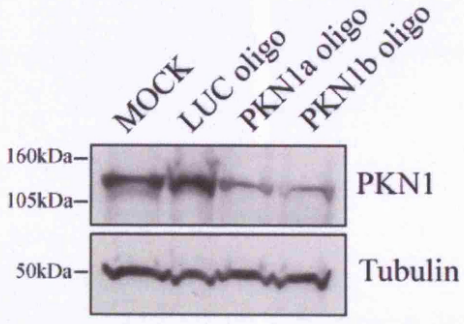
an in-house rabbit polyclonal antibody (see Figure 4:1) this antibody no longer enabled detection of a specific PKN3 protein band. A new batch of anti-sera was purified to attempt to overcome this problem but again no protein bands were detected at any antibody concentration or incubation conditions. However it is known that the siRNA oligo used to target PKN3 is capable of achieving knockdown as reported by Leenders et al who used a rabbit polyclonal antibody they had raised against PKN3 to check PKN3 knockdown in PC3 cells (Leenders et al., 2004). Additionally Dr Sylvie Lachmann in our lab showed that the PKN3 siRNA oligo was capable of depleting the levels of overexpressed GFP-PKN3 (see Figure 4:4). Therefore in the absence of a stable in-house anti-sera or a good commercial antibody for PKN3 it was assumed that if depletion of PKN1 and PKN2 was achieved then the transfection of the siRNA oligos was successful and hence PKN3 levels would also have been depleted albeit the extent could not be confirmed. The average results of four assays comparing the effect of PKN isoform depletion on migration relative to a LUC control are shown in (Figure 4:3). Depletion of the PKN isoforms was seen to result in a statistically significant (30%) decrease in migration compared to the LUC control.

These results suggest that the PKNs contribute to cell migration in a cell type dependent manner since in MCF7 cells where PKN1 is the predominant isoform knockdown of PKN1 does not affect the speed of migration. However in MDAMB-468 cells that express all three PKN isoforms combined knockdown of the isoforms results in a 30% decrease in the speed of migration relative to the LUC control. However it can not be conclusively stated that the PKN isoforms contribute to cell migration in the MDAMB-468 cells since there is a possibility that the siRNA oligos are having off-target effects (Jackson and Linsley, 2004), this consideration and possible further validation experiments are discussed in more detail in chapter 6.

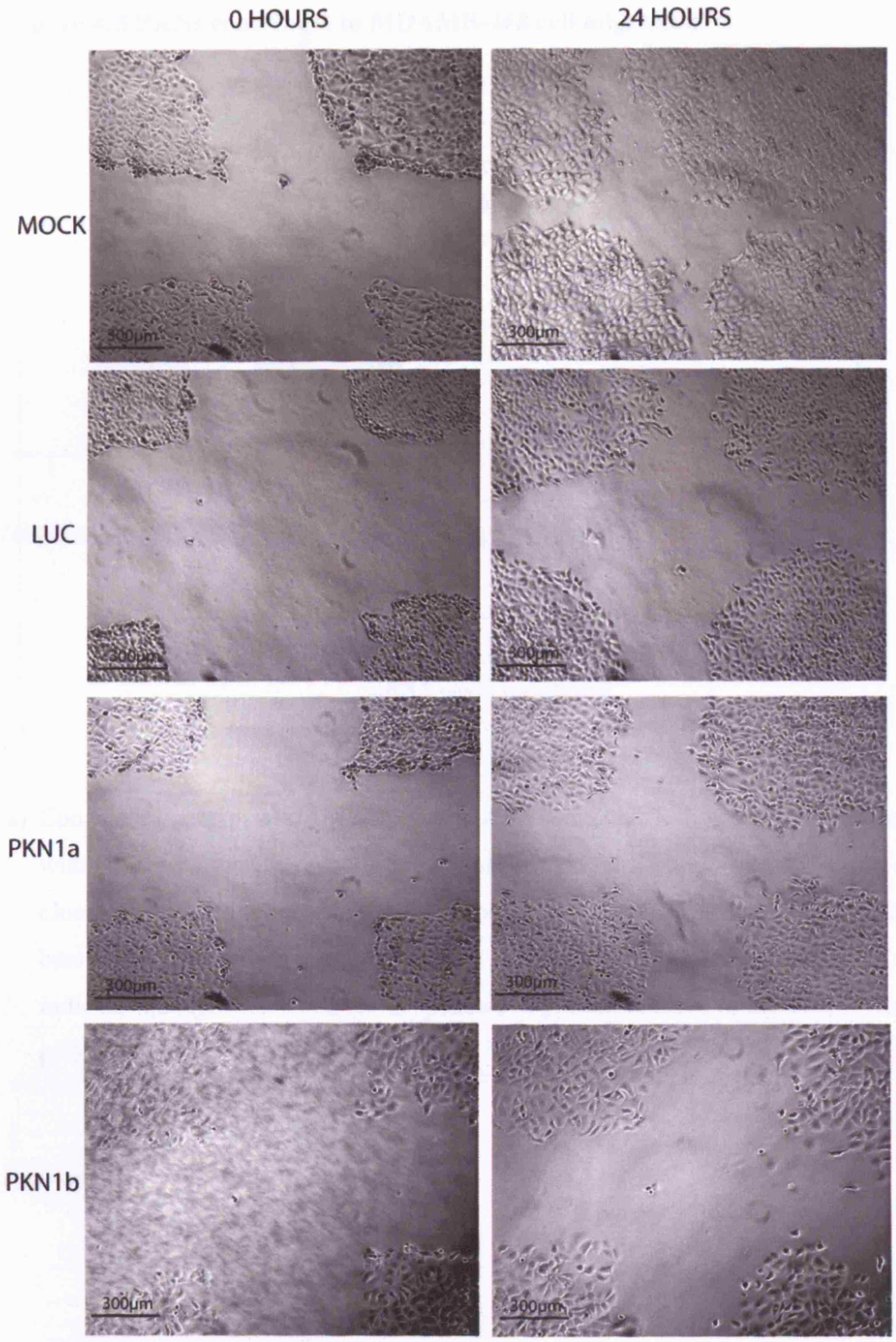
Figure 4:2 PKN1 depletion does not affect MCF7 cell migration.



a) Confluent layers of MCF7 cells were scratched 72 hours after transfection with siRNA oligos. The wound areas were filmed for 24 hours and the speed of closure calculated relative to the LUC controls, n=3 performed in triplicate and the error bars show the standard error. Bonferroni corrected Z tests were performed; n.s. indicates no significant change in speed of migration relative to control.

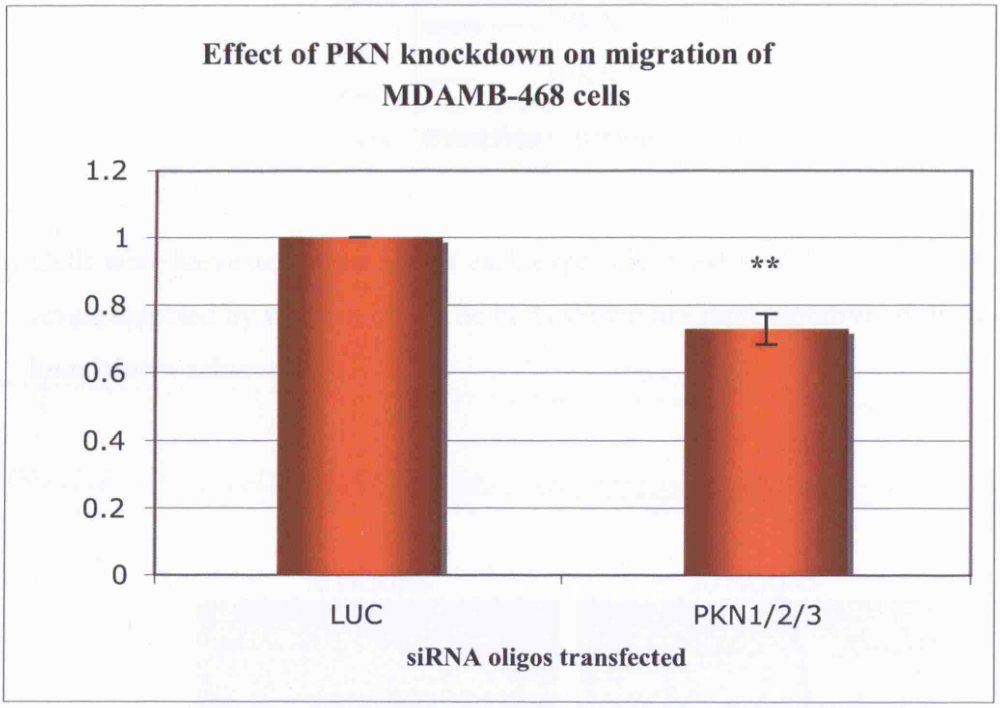


b) Cells were harvested at the end of each experiment and PKN1 and tubulin levels detected by western blot. The blots shown are representative of the levels of PKN1 knockdown achieved.

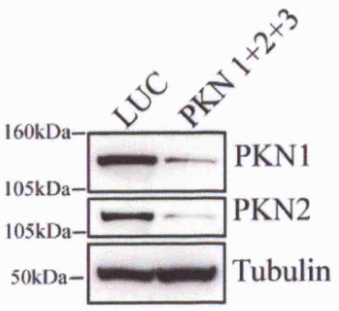


c) Representative phase contrast images of MCF7 cells at the beginning and end of a scratch wound assay. Scale bars are equivalent to 300µm.

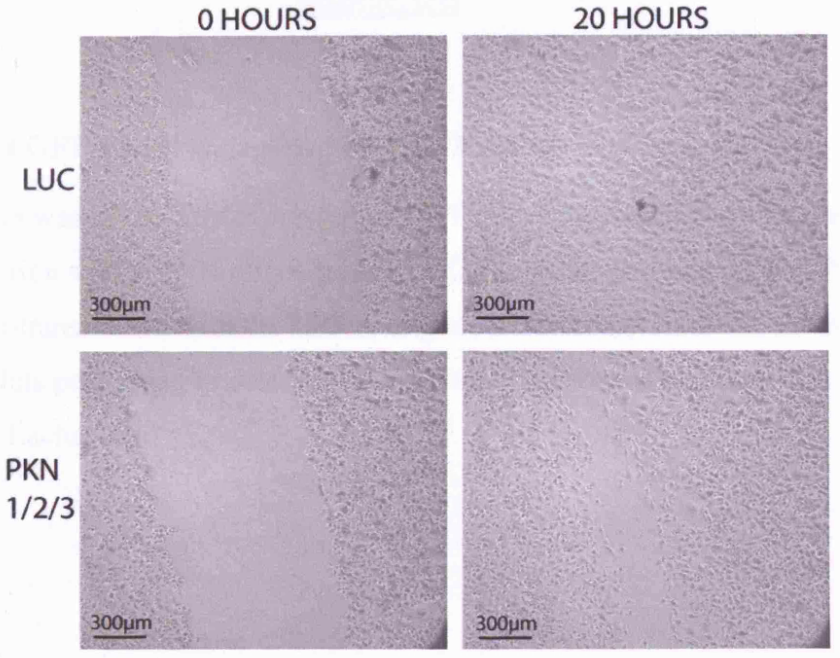
Figure 4:3 PKNs contribute to MDAMB-468 cell migration.



a) Confluent layers of MDAMB-468 cells were scratched 72 hours after transfection with siRNA oligos. The wound areas were filmed for 24 hours and the speed of closure calculated relative to the LUC controls; n=4, performed in triplicate the error bars show the standard error. Bonferroni corrected Z tests were performed; n.s. indicates no significant change in speed of migration relative to control; ** represents $p<0.01$.



b) Cells were harvested at the end of each experiment and PKN1, PKN2 and tubulin levels detected by western blot. The blots shown are representative of the levels of knockdown achieved.



c) Representative phase contrast images of MDAMB-468 cells at the beginning and end of a scratch wound assay (see Appendix for movie files). Scale bars are equivalent to 300µm.

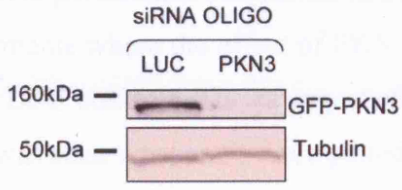


Figure 4:4 GFP-PKN3 is depleted by PKN3 siRNA oligo.

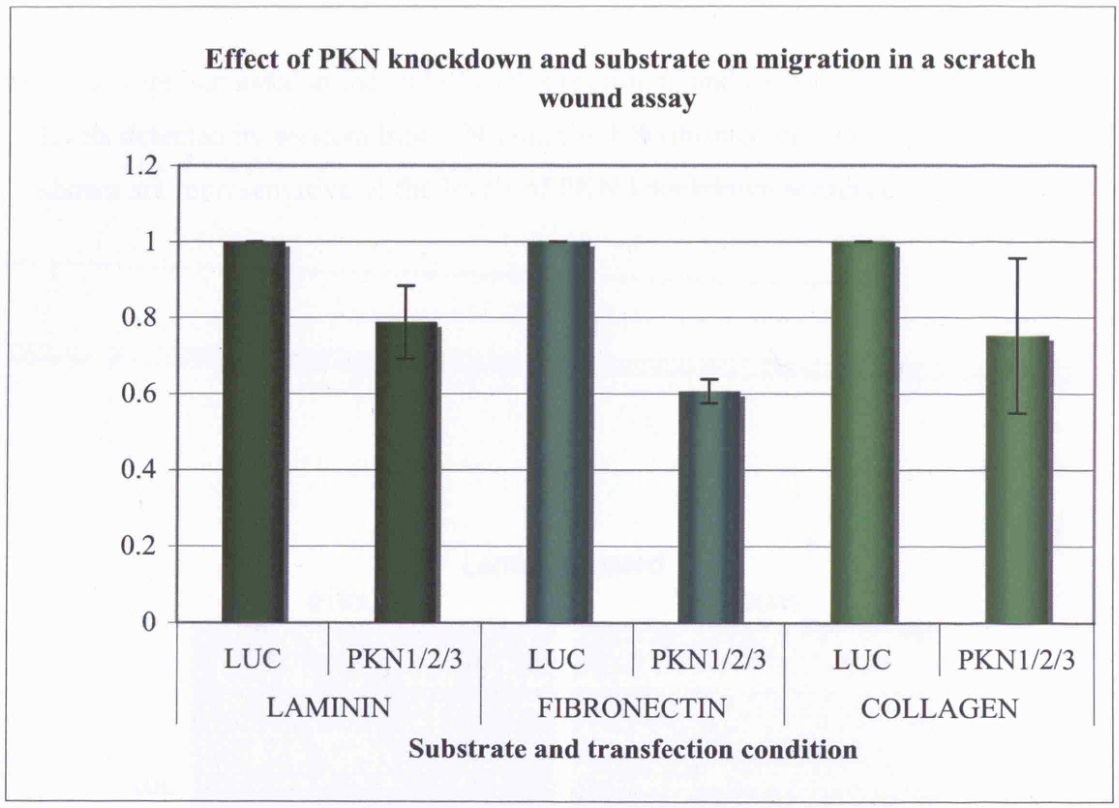
GFP-PKN3 was transiently expressed in 5637 cells (transformed bladder cell line) prior to transfection with siRNA oligos (either LUC control or targeted against PKN3). 72 hours post-transfection with the siRNA oligos cells were harvested in sample buffer and western blots performed to detect PKN3 and tubulin levels. This work was performed by Dr Sylvie Lachmann.

4.3 The role of PKN in 2-D migration of MDAMB-468 cells is substrate specific.

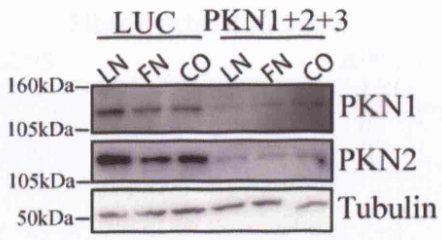
Since the PKNs appear to contribute to the ability of MDAMB-468 cells to migrate it was decided to test whether the nature of the matrix-cell interaction had any effect on this involvement. Cells make contacts with the underlying substrate via cell surface receptors such as integrins (see 1.1.2). In order to assess whether the PKNs signalled downstream of specific integrins the effect of PKN depletion on migration on different substrates was examined. Cells were plated on laminin, fibronectin or collagen and scratch wound assays were performed. The results shown in Figure 4:5a are an average of three separate experiments where the effect of PKN depletion on speed of migration is shown relative to the LUC controls. The strongest effect of depletion of PKN isoforms on migration was seen when cells were plated on fibronectin. Under these conditions a 40% decrease in migration was observed. Pre-coating the plates with collagen resulted in a greater variability in migration between assays and makes the 30% decrease in migration observed not significant. Pre-coating the plates with laminin resulted in only a 20% decrease in migration due to PKN depletion. In Figure 4:3 it can be seen that cells migrating on untreated plates displayed a 30% decrease in migration upon PKN isoform depletion. However since these assays were carried out in the presence of 10% foetal calf serum and fibronectin is a common matrix constituent of serum it is likely that the same integrin receptors are engaged in this condition as when the plates were pre-coated with fibronectin.

These results suggest that the PKN contribution to cell migration is dependent on the extracellular ligands present and hence the cell surface receptors that are engaged.

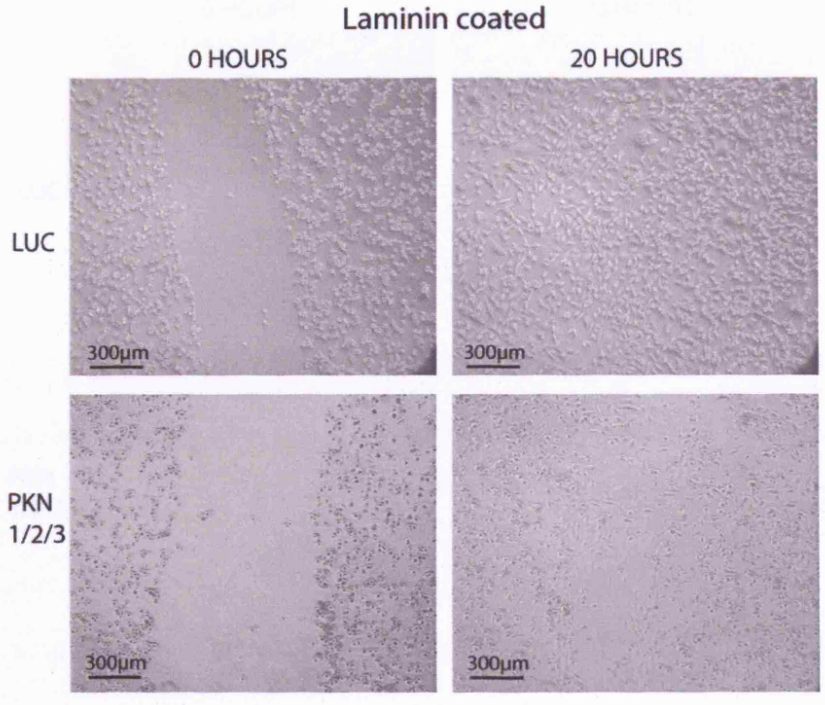
Figure 4:5 Substrate affects the contribution of PKN isoforms to migration of MDAMB-468 cells.

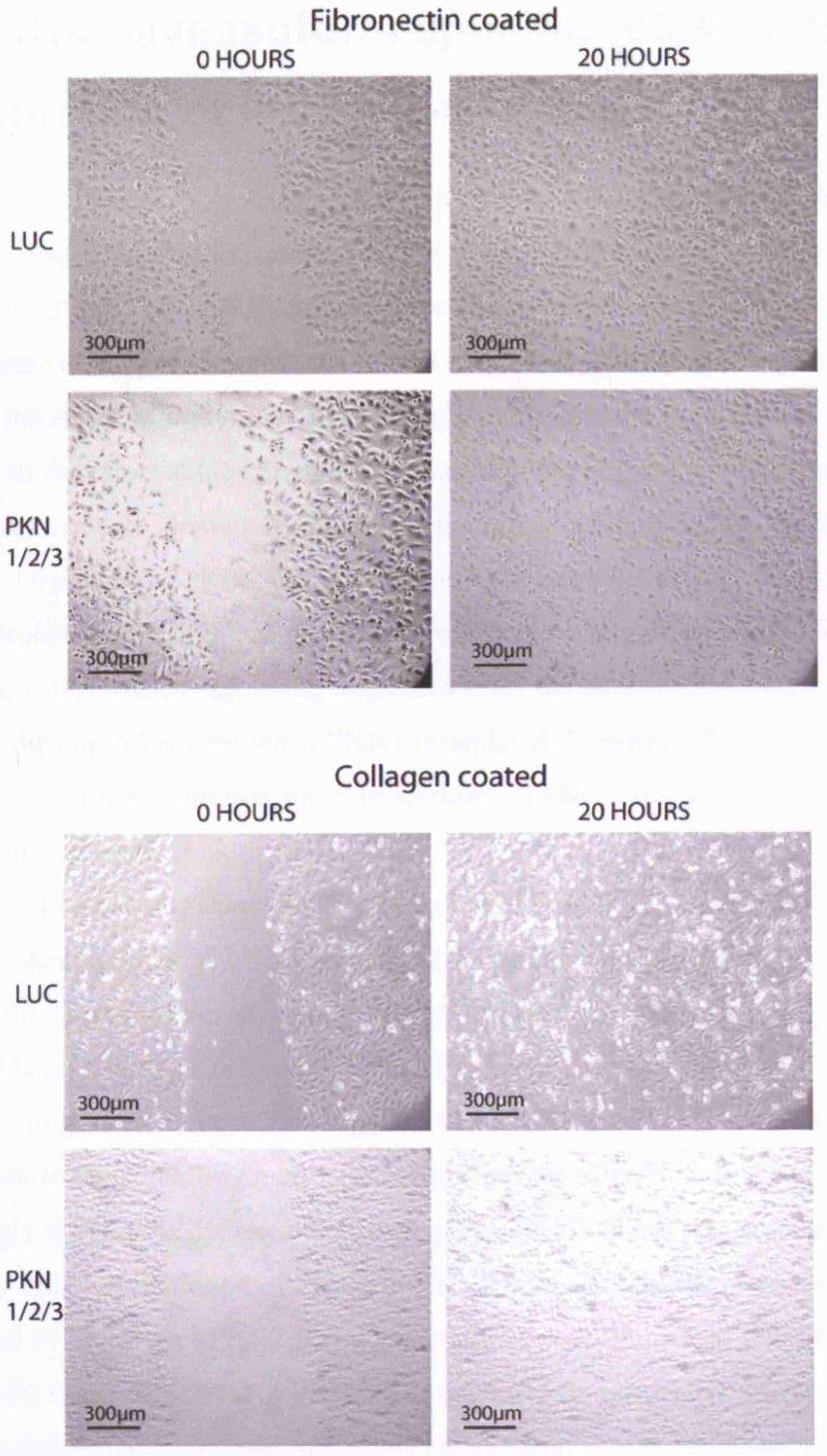


a) Confluent layers of MDAMB-468 cells were scratched 72 hours after transfection with siRNA oligos. The wound areas were filmed for 24 hours and the speed of closure calculated relative to the LUC controls; n=2 performed in triplicate and the error bars show the standard error.



b) Cells were harvested at the end of each experiment and PKN1, PKN2 and tubulin levels detected by western blot. LN-laminin, FN-fibronectin, CO-collagen. The blots shown are representative of the levels of PKN knockdown achieved.





c) Representative phase contrast images of MDAMB-468 cells at the beginning and end of a scratch wound assays performed on different substrates (see Appendix for movie files). Scale bars are equivalent to 300µm.

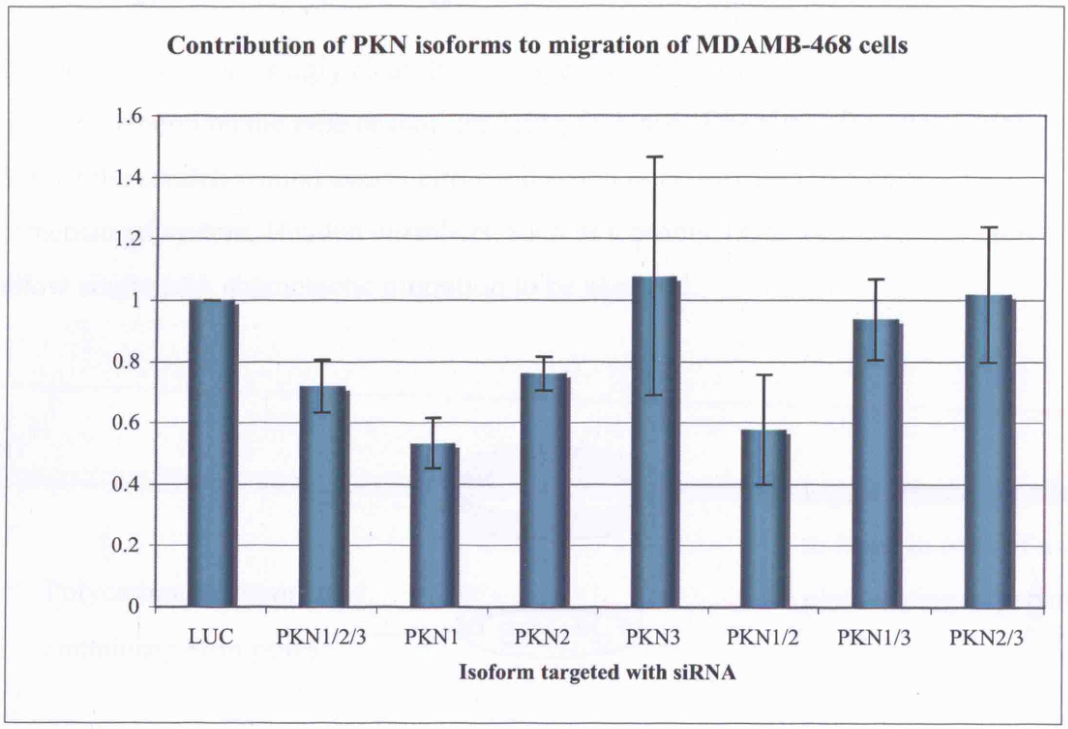
4.4 PKNs have isoform specific roles in the 2-D migration of MDAMB-468 cells.

Having shown that the PKN isoforms can play a role in the migration of MDAMB-468 cells it was logical to next assess the relative contribution of each PKN isoform. In a scratch wound assay on uncoated plates knockdown of all three isoforms results in a 30% decrease in migration compared to a LUC control. The same assay was employed to look at the effect of individual isoform and double isoform knockdown on migration compared to the effect achieved with triple knockdown (Figure 4:6). Consistent with the previous experiments, depletion of all three isoforms results in a 30% decrease in migration. Depletion of PKN1 or PKN2 alone has a negative effect on migration whereas depletion of PKN3 has no significant effect on migration. PKN1 apparently plays the greatest role in regulating migration since the largest reduction in migration relative to the control is seen when PKN1 is depleted. However PKN1 cannot compensate for PKN2 function since knockdown of PKN2 still results in a 25% reduction in migration.

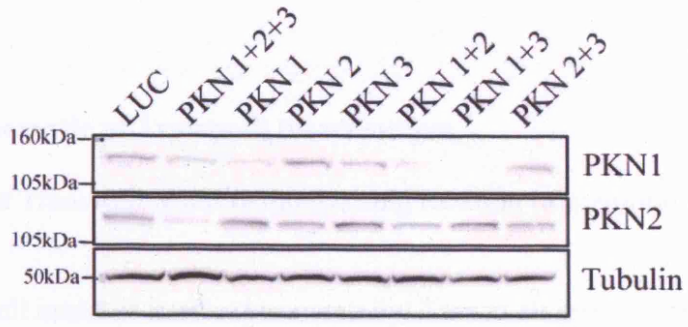
The results of combining knockdown of two isoforms at the same time are particularly interesting. Knockdown of PKN1 and PKN2 might have been expected to have an additive effect on migration compared to the 50% decrease seen with PKN1 knockdown and the 25% seen with PKN2 knockdown. However this is not the case and only a 50% decrease in migration is seen. This suggests that PKN1 and PKN2 have overlapping roles to play in the signalling pathways controlling the migration of these cells. Interestingly if PKN3 is depleted alongside either PKN1 or PKN2 then there is no longer any effect of depletion of PKN1 or PKN2 on migration despite similar levels of knockdown of PKN1 and PKN2 being achieved. This suggests that PKN3 knockdown overrides the negative effects of PKN1/2 knockdown on migration. Consistent with PKN3 knockdown having a positive effect on migration is the observation that knockdown of all three isoforms together results in a 30% decrease in migration whereas knockdown of PKN1 alone gives a 50% decrease in migration.

These results suggest that although PKN1 plays the greatest role of the three PKN isoforms in controlling migration all of the isoforms make a contribution to regulating migration in a scratch wound assay in MDAMB-468 cells.

Figure 4:6 PKN1 and PKN2 play a role in the migration of MDAMB-468 cells.



a) Confluent layers of MDAMB-468 cells were scratched 72 hours after transfection with siRNA oligos. The wound areas were filmed for 24 hours and the speed of closure calculated relative to the LUC controls; n=2 performed in triplicate and the error bars show the standard error.



b) Cells were harvested at the end of each experiment and PKN1, PKN2 and tubulin levels detected by western blot.

4.5 Substrate specific role for PKN isoforms in migration through a Transwell

It is becoming increasingly clear that the signalling pathways activated during cell motility depend on the type of motility being assessed (Friedl and Brocker, 2000). Whilst the scratch wound assays allow migration to be assessed in a collective 2-dimensional system, Boyden chambers, such as Corning Transwell inserts (Figure 4:7), allow single cell, chemotactic migration to be assessed.

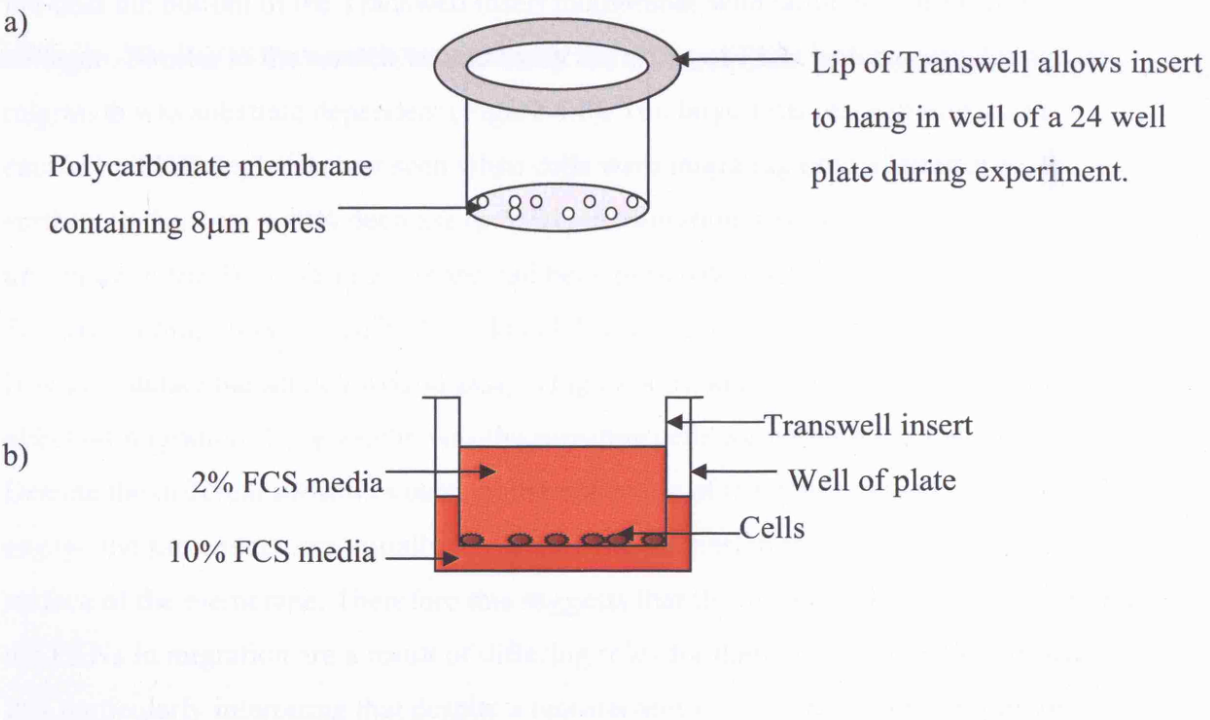


Figure 4:7 Schematic of Transwell insert system.

a) Diagram of a Transwell insert demonstrating location of membrane containing 8μm pores.

b) The Transwell insert as used experimentally. Transwell insert is placed into a well of a 24 well plate, media added to the well and the insert and cells seeded onto the top of the Transwell insert membrane.

Seeding cells directly onto the upper membrane of a Boyden chamber provides another assessment of the ability of cells to migrate albeit under different constraints to those imposed by the scratch wound assay previously used to investigate the PKNs contribution to cell migration. In the Boyden chamber migration assay cells must adhere to the upper surface of the membrane prior to migrating as single cells through the pores in the membrane towards a serum gradient.

To investigate if PKN isoforms play a role in the migration of MDAMB-468 cells in this type of migration assay, cells were again depleted of all three PKN isoforms using siRNA. Since substrate had an effect on the role that PKN had to play in migration in scratch wound assays and to help aid migration through the membrane it was decided to pre-coat the bottom of the Transwell insert membranes with laminin, fibronectin or collagen. Similar to the scratch wound assay the effect of PKN isoform knockdown on migration was substrate dependent (Figure 4:8). The largest decrease in migration caused by PKN depletion was seen when cells were migrating onto a laminin coated surface, in this case a 40% decrease ($p<0.01$) in migration was observed. If the underside of the Transwell membrane had been pre-coated with collagen then the decrease in migration caused by PKN knockdown was approximately 30% ($p<0.01$). However unlike the scratch wound assay, (Figure 4:5), knockdown of PKN had no effect on migration if fibronectin was the substrate cells were migrating towards. Despite the different substrates used on the underside of the membrane all cells should engage the same receptors initially to regulate their adhesion to the uncoated upper surface of the membrane. Therefore this suggests that the different requirements seen for the PKNs in migration are a result of differing roles for them in chemotactic motility. It is particularly interesting that despite a requirement for the PKNs in migration on fibronectin there is no requirement for them in migration towards fibronectin. It was shown previously that in a scratch wound assay the PKN isoforms had different roles to play in controlling migration. The possibility is that the PKN isoforms also play different roles in migration in the Boyden chamber migration system.

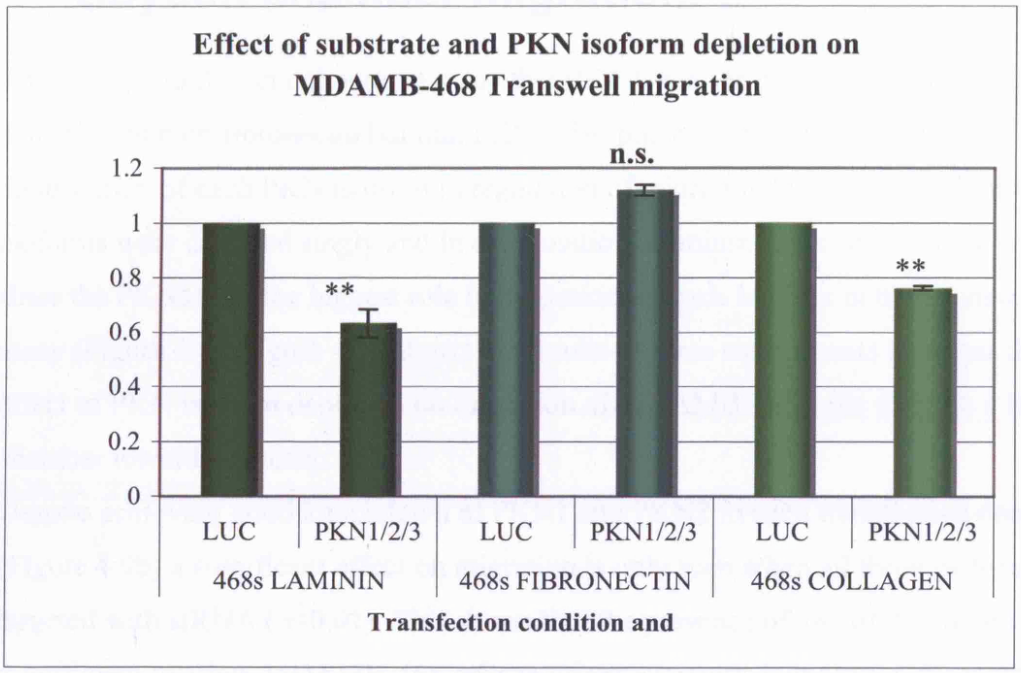


Figure 4:8 Effect of PKN depletion on migration through a Boyden chamber is substrate dependent.

72 hours post-transfection cells were harvested and counted. 5 x 10^4 cells were seeded into each Boyden chamber (which had been precoated with substrate on the underside of the membrane) and allowed to migrate for 5 hours. The number of cells migrated through to the underside was counted and compared relative to the LUC control; n=3, performed in triplicate the error bars show the standard error. Bonferroni corrected Z tests were performed; n.s. indicates no significant change in migration relative to control; ** represents $p<0.01$.

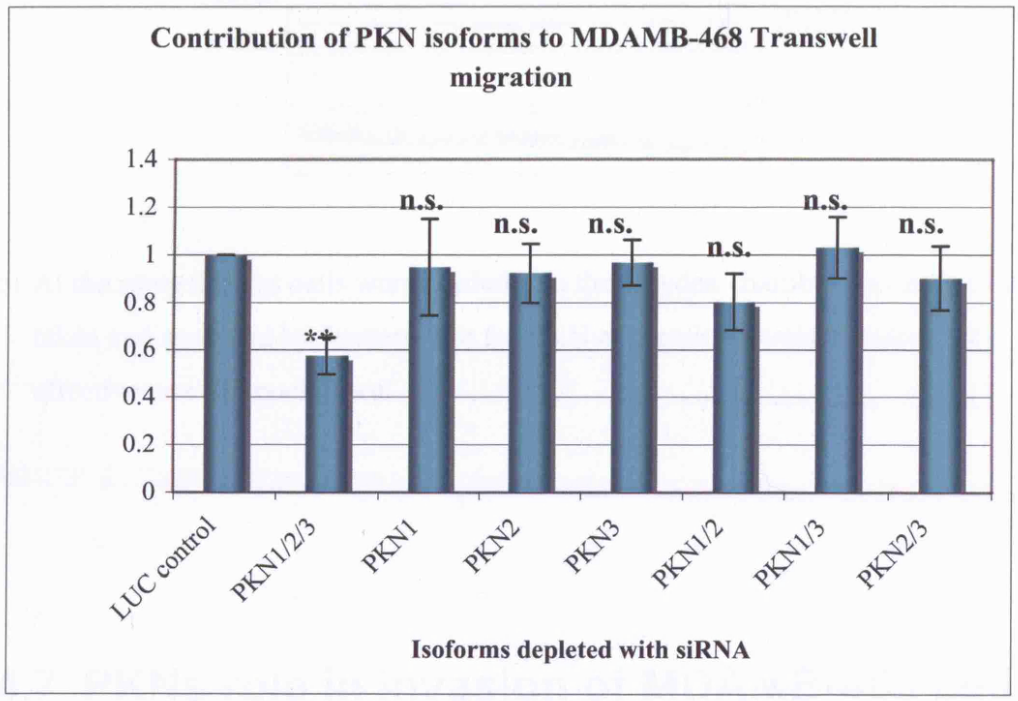
4.6 PKN isoforms show redundant function in Boyden chamber migration.

It was found in the scratch wound assay that PKN1 was the predominant regulator of cell migration on fibronectin but that PKN2 also played a role. To examine the contribution of each PKN isoform in regulation of migration towards laminin the isoforms were depleted singly and in combination. Laminin was chosen as the substrate since the PKNs play the biggest role in migration towards laminin in the Transwell assay (Figure 4:8). Figure 4:9a shows the results of three experiments to assess the effect of PKN isoform depletion on migration of MDAMB-468 cells through a Boyden chamber towards laminin.

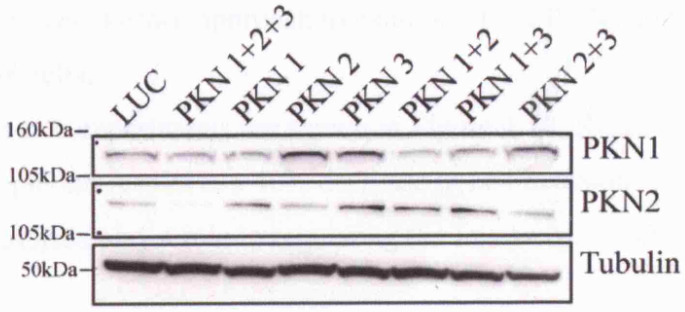
Despite achieving good knockdown of PKN1 and PKN2 in each transfection condition (Figure 4:9b) a significant effect on migration is only seen when all three isoforms are targeted with siRNA ($p<0.01$). This shows that the presence of any of the three isoforms is sufficient to allow MDAMB-468 cells to migrate towards laminin i.e. the isoforms have redundant functions in this migration assay. This could be due either to upregulation of the signalling pathways which the remaining isoform(s) act on, or because they all lie on the same pathway. The finding that the PKN isoforms all have to be depleted in this assay for an effect on migration to be seen suggests that the oligos are not having an off-target effect on a key regulator of cell migration.

The results from the Boyden chamber and scratch wound migration assays suggest that PKN function in cell motility is different depending on the environment and the nature of the cell migration that is being assessed. Another important mode of cell motility in cancer is invasion (see 1.7.2) and the contribution of the PKN isoforms to this mode of cell motility was investigated next.

Figure 4:9 PKN isoforms display redundancy of function in migration towards laminin.



a) 72 hours post-transfection cells were harvested and counted. 5 x 10^4 cells were seeded into each Boyden chamber (which had been precoated with substrate on the underside of the membrane) and allowed to migrate for 5 hours. The number of cells migrated through to the underside was counted and compared relative to the LUC control; n=3, performed in triplicate the error bars show the standard error. Bonferroni corrected Z tests were performed; n.s. indicates no significant change in speed of migration relative to control; ** represents $p<0.01$.



b) At the same time as cells were seeded into the Boyden chamber a sample of each was taken and analysed by western blot for PKN expression levels to assess the effectiveness of knockdown.

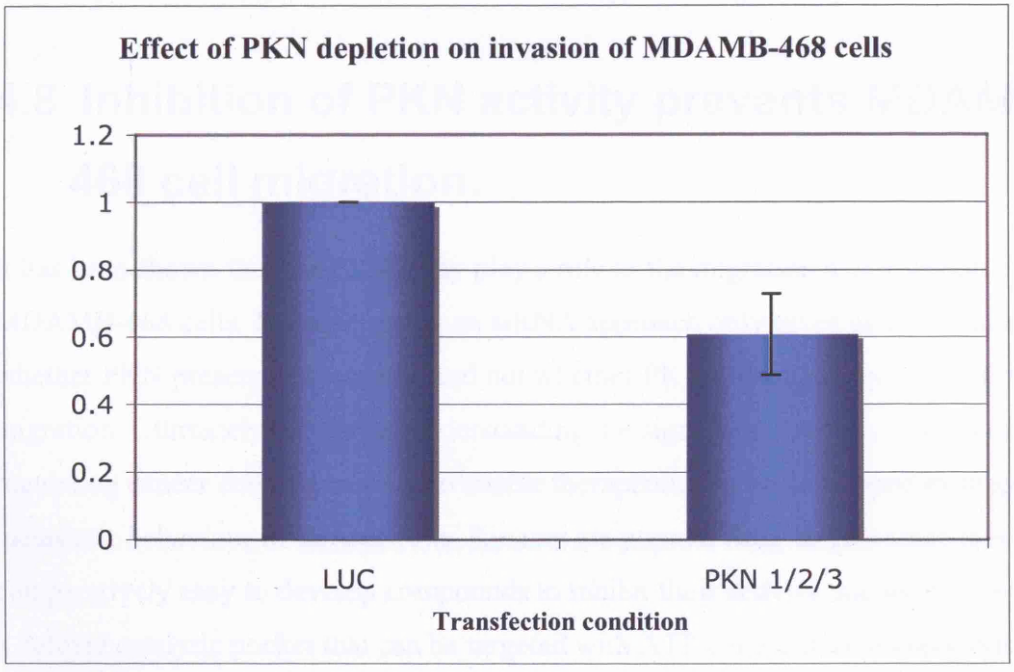
4.7 PKNs role in invasion of MDAMB-468 cells.

By seeding cells into Boyden chambers that have been coated with a thin layer of extracellular matrix proteins the ability of cells to move through this matrix can be examined giving a measure of the invasive capability of the cells. Growth factor reduced Matrigel is commonly used to assess invasion in this system and serves as a reconstituted basement membrane *in vitro.* According to the manufacturer its major constituent is laminin followed by collagen IV and other extracellular matrix components. Cells are seeded on top of this layer and must move through the matrix to reach the pores in the membrane. Cells that have reached the underside of the membrane after a certain time period are then counted. This assay was used to investigate whether the PKNs contribute to the invasive phenotype of MDAMB-468 cells by examining the effect of depletion of the three PKN isoforms. Similarly to the migration assays comparison of the number of cells that invade after transfection with a Luciferase control siRNA oligo to the number of cells that invade after knockdown of the PKN isoforms was used to investigate PKN involvement in cell invasion. Since in the Boyden chamber migration assay the PKN isoforms displayed redundant function it was decided

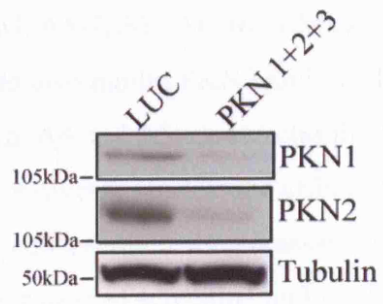
to use a combined knockdown approach to examine if the PKNs contribute to invasion of MDAMB-468 cells.

The results from two experiments are shown in Figure 4:10. Relative to the LUC control, PKN depletion results in a 40% decrease in cell invasion. This result suggests that the PKN isoforms play a role in regulating the invasion of MDAMB-468 cells *in vitro*.

Figure 4:10 Effect of PKN knockdown on invasion of MDAMB-468 cells.



a) 72 hours post-transfection cells were harvested and counted. 5 x 10^4 cells were seeded into each Boyden invasion chamber and allowed to invade for 48 hours. The number of cells on the underside of the membrane after this time was counted and compared relative to the LUC control, experiments performed in quadruplicate, n=2, the error bars display the standard error.



b) At the same time as cells were seeded into the Boyden chamber a sample of each was taken and analysed by western blot for PKN expression levels to assess the effectiveness of knockdown

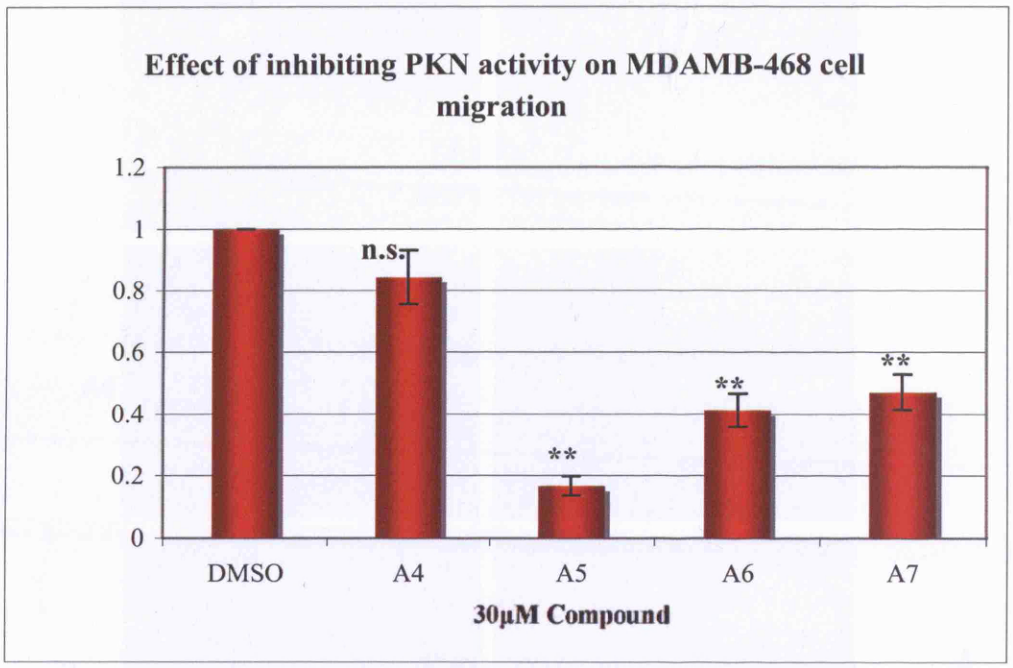
4.8 Inhibition of PKN activity prevents MDAMB-468 cell migration.

It has been shown that the PKNs may play a role in the migration and invasion of MDAMB-468 cells. However using an siRNA approach only gives us information on whether PKN presence is required and not whether PKN activity is necessary for migration. Ultimately the aim of understanding the signalling pathways involved in regulating cancer cell migration is to enable therapeutics to be developed to suppress metastatic behaviour of tumour cells. Kinases are popular drug targets since it is comparatively easy to develop compounds to inhibit their activity due to the presence of a defined catalytic pocket that can be targeted with ATP competitive compounds (Fabbro et al., 2002). In collaboration with Cancer Research Technology a library screen of small molecular weight compounds was undertaken to find novel PKN inhibitors. This resulted in three related hits; one related compound that did not inhibit PKN1 or PKN2 was chosen as a control. This collaboration is still ongoing and due to patenting restrictions the structures of these compounds can not be disclosed. The compounds will be referred to as A4 (the control), A5, A6 and A7. The IC50 values for PKN1 of these

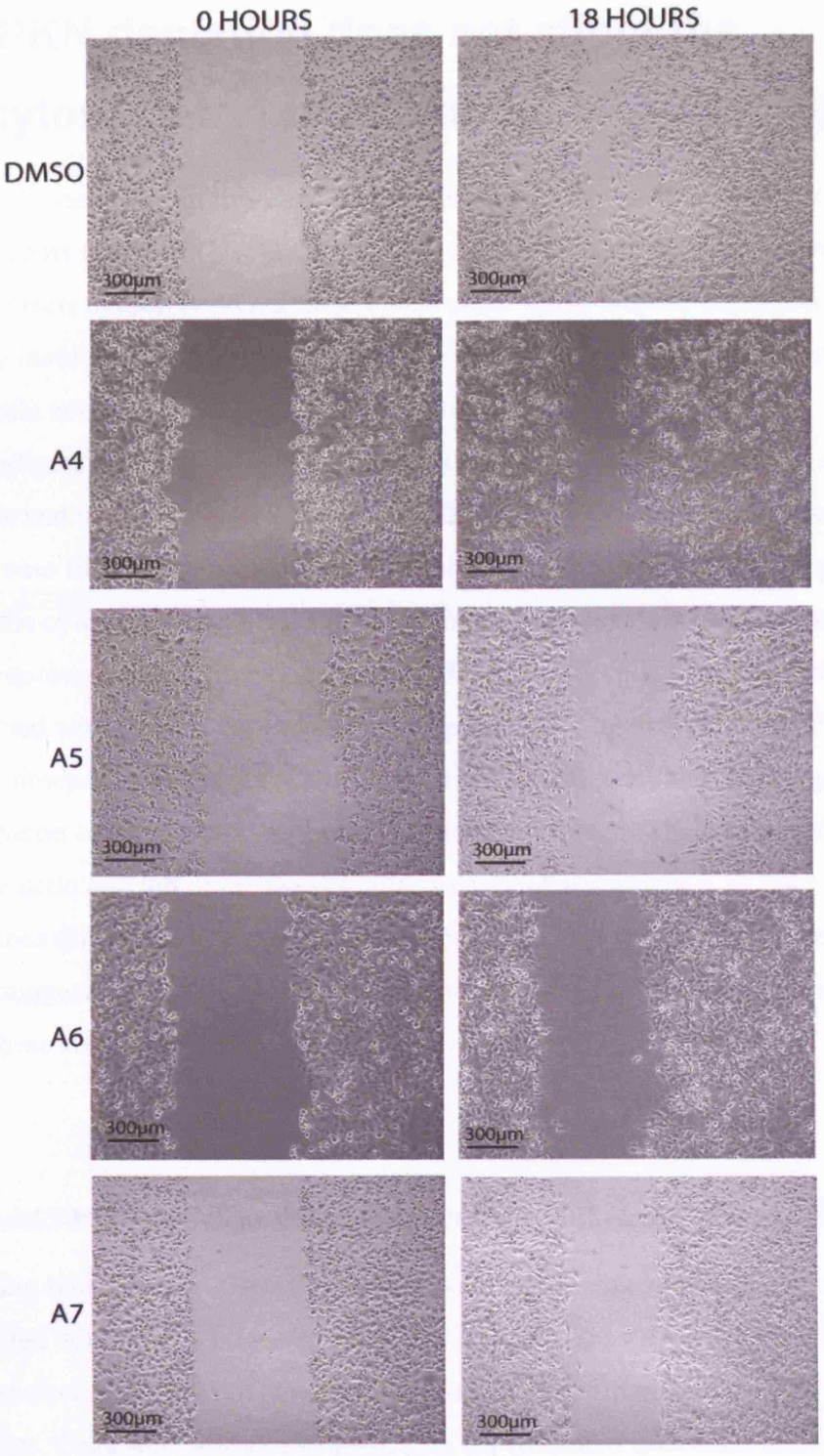
compounds are; A4 >100μM, A5-7μM , A6-16.5μM and A7-3.5μM. The compounds A5, A6 and A7 are known to also inhibit PKN2 and PKN3 but the cell permeability of the compounds is not known. A4 and A5 are structurally very highly related compounds whereas A6 and A7 are more diverse. To test the effect of inhibiting PKN activity on migration of MDAMB-468 cells, scratch wound assays were performed blind ±inhibitor. This assay was chosen for the ease of screening and to allow any effects of the compounds on cell viability to be observed.

All three active compounds significantly decreased migration ($p<0.01$) by at least 55% when used at 30μM concentration whereas the inactive control A4 compound had no significant effect on migration decreasing migration by only 15% when compared to DMSO alone (Figure 4:11). A7 was observed to be mildly toxic to the cells over the time course of the assay but none of the other compounds were toxic to the cells, as judged by the extent of cell detachment from the substrate, at 30μM (see Appendix for movie files). These results suggest that the catalytic activity of the PKNs is required for MDAMB-468 cell migration although it is possible that kinases other than the PKNs that also contribute to cell migration are also inhibited by these compounds.

Figure 4:11 Inhibiting PKN activity decreases migration of MDAMB-468 cells.



a) Confluent layers of MDAMB-468 cells were pre-treated with 30μM of the relevant compound for 1 hour prior to wounding. Migration into the wound area in the presence of 30μM compound was filmed over a period of 24 hours and the time taken to close the wound compared to a DMSO control. The average speed of wound closure standardised to the DMSO control is shown and the error bars display the standard error; n=3 performed in triplicate; a Bonferroni corrected Z test was performed, n.s shows that no significant change in speed was observed, ** shows $p<0.01$.



b) Representative phase contrast images of MDAMB-468 cells at the beginning and end of a scratch wound assays performed in the presence of CRT compounds or DMSO as a vehicle control (see Appendix for movie files). Scale bars are equivalent to 300µm.

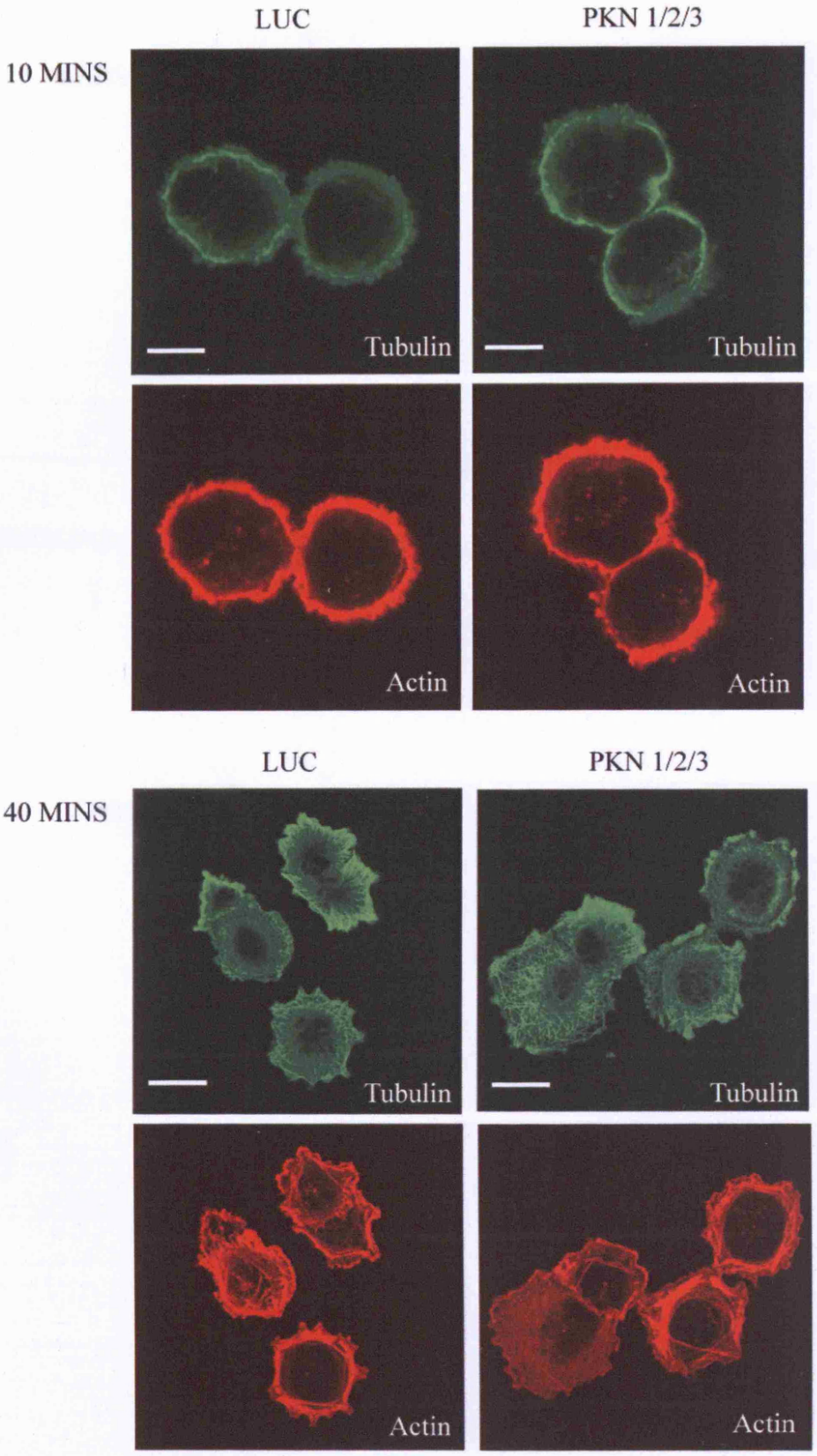
4.9 PKN depletion does not affect the cytoskeleton of spreading MDAMB-468 cells.

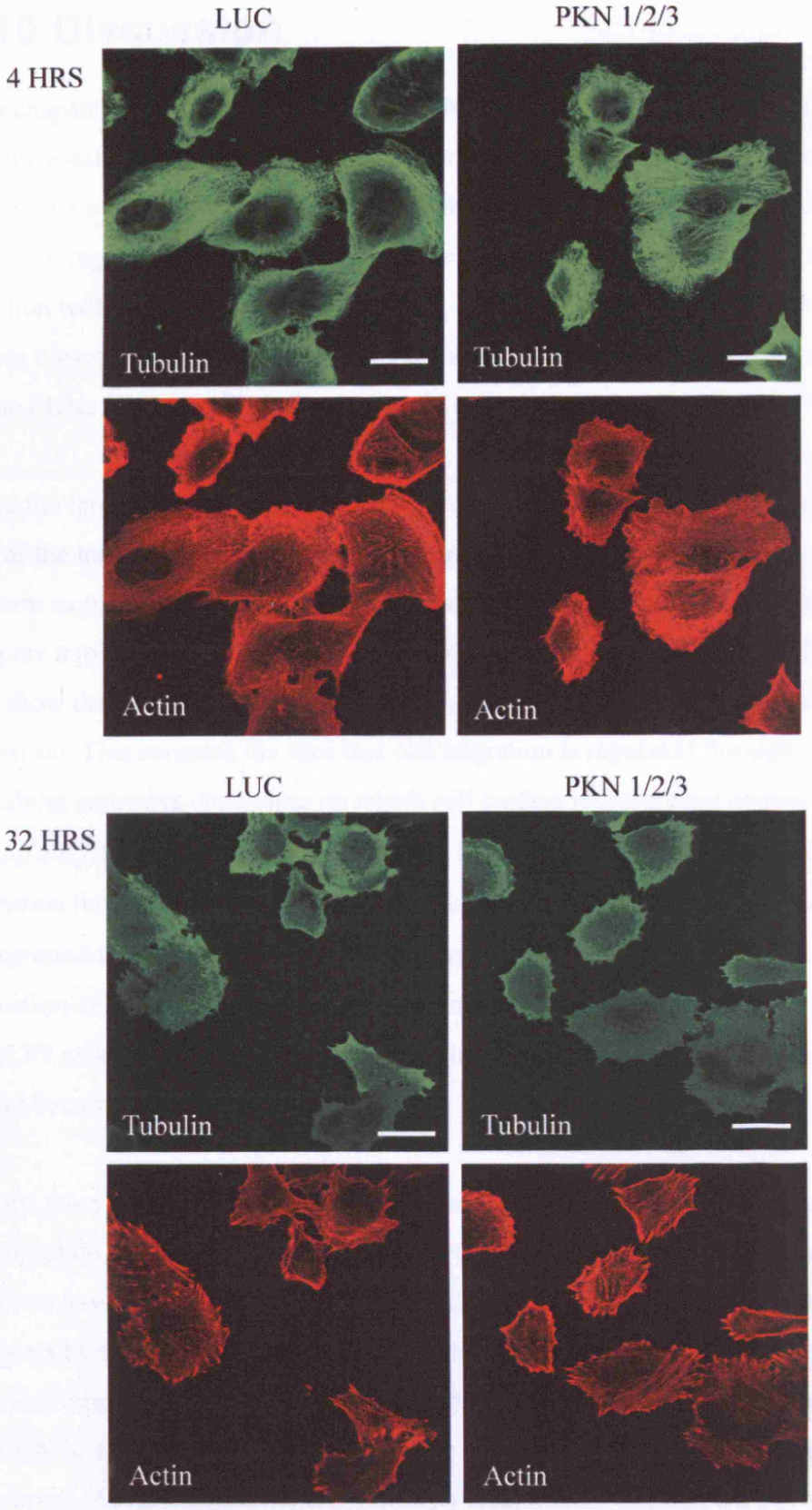
The results described in this chapter have indicated substrate and motility specific requirements for the PKNs. In Chapter 3 it was shown that overexpression of GFP-PKN1 affects cytoskeleton rearrangements under hyperosmotic conditions. Since cell motility involves dynamic regulation of the cytoskeleton it is possible that the PKNs play a role in these cytoskeleton rearrangements.

To investigate this possibility it was decided to look at the effect of PKN depletion on the actin and microtubule cytoskeleton. Cells were harvested 72 hours post transfection, seeded onto fibronectin coated glass coverslips and fixed at various time points to enable the cytoskeleton to be clearly visualised under dynamic conditions. Figure 4:12 shows representative confocal images of MDAMB-468 cells that have either been transfected with the Luc control siRNA oligo or oligos against the three PKN isoforms. Several timepoints after seeding of the cells are shown to allow the changes in the cytoskeleton to be observed as the cells adhere and spread. The pictures show that whilst both the actin and tubulin networks both undergo changes over a 32 hour period there is no obvious difference in either network between control and PKN depleted cells. These results suggest that the PKN isoforms do not play a role in cytoskeleton remodelling under these conditions.

Figure 4:12 PKN depletion does not affect the cytoskeleton of spreading cells.

Following two pages: MDAMB-468 cells were either control transfected (LUC) or transfected with siRNA oligos against PKN1, PKN2 and PKN3 (PKN1/2/3). 72 hours post-transfection cells were harvested and seeded onto fibronectin coated glass coverslips. Cells were allowed to adhere for the indicated times and then fixed. The actin and microtubule cytoskeleton was visualised by staining with phalloidin and anti-tubulin antibody. The confocal images are a 1μm 'Z' optical sections and are representative of three independent experiments. The scale bar is equivalent to 10μm.


LUC
PKN 1/2/3
10 MINS
Tubulin
Tubulin
Actin
Actin
LUC
PKN 1/2/3
40 MINS
Tubulin
Tubulin
Actin
Actin


LUC
PKN 1/2/3
4 HRS
Tubulin
Tubulin
Actin
Actin
LUC
PKN 1/2/3
32 HRS
Tubulin
Tubulin
Actin
Actin

4.10 Discussion

This chapter set out to look at whether PKN1 plays a role in transformed cell motility and the results described suggest that under certain circumstances PKN1 contributes to the regulation of cell migration. However the results also suggest that PKN1 is not a universal regulator of cell motility and indeed under some conditions has overlapping function with the two other PKN isoforms. Whilst the PKNs were found to be expressed across a spectrum of transformed cells it was decided to focus on investigating the role of the PKNs in the motility of transformed cells derived from tumours of the breast.

Since the interest of this thesis is primarily the PKN1 isoform the MCF7 cell line was one of the transformed breast cell lines chosen to study since PKN1 is the predominant isoform expressed in this cell line. Use of siRNA technology suggested that PKN1 does not play a role in MCF7 cell migration. However results from the MDAMB-468 cell line show that the substrate cells are plated on affects whether the PKN isoforms control migration. This supports the idea that cell migration is regulated through different signalling pathways depending on which cell surface receptors are engaged. For example it has been shown that fibronectin binding β1 integrins promote random migration through extension of multiple thin protrusions whereas in the same cell background fibronectin binding β3 integrins promote persistent migration through the formation of a single broad lamellipod (Danen et al., 2005). It is therefore possible that if MCF7 cells were plated on a different substrate then a role for PKN1 in cell motility would become evident.

Results from the MDAMB-468 cell line are intriguing since the substrate specific effects on migration vary according to the motility assay used. In the scratch wound assay PKN isoforms have the greatest effect on migration on fibronectin since depletion of the isoforms by siRNA results in a 40 % reduction in migration. However in the Boyden chamber migration assay when the underside of the membrane is coated in fibronectin the PKN isoforms play no role in migration of the cells to the coated surface of the membrane. At first glance these contrasting results seem unexpected but given that the

two assays are measuring fundamentally different types of cell motility it is perhaps not surprising that there are some differences in requirements for the PKNs. In the Boyden chamber assay cells are chemotaxing and migrating as individual cells towards a serum and substrate gradient. In contrast, in the scratch wound assay, as well as cell-matrix contacts the cells also have substantial cell-cell contacts that must be regulated to co-ordinate migration of groups of cells. Despite the lack of involvement of the PKNs in migration towards fibronectin they do play a role in migration of the MDAMB-468 cells towards both laminin and collagen where respectively a 40% and 25% decrease in migration is seen upon knockdown of the PKNs.

Isoform specific requirements also differed between the two migration assays used. In the Transwell assay the isoforms were found to have redundant function in that in order for a decrease in migration to be seen all three isoforms had to be targeted by siRNA. Knockdown of any single isoform or combination of two isoforms did not result in a decrease in migration. However in the scratch wound assay when cells are migrating on fibronectin knockdown of PKN1 or PKN2 was found to have a negative effect on migration whereas PKN3 knockdown decreased the effect of PKN1 or PKN2 knockdown. Since knockdown of PKN3 did not affect the level of depletion of PKN1 or PKN2 that was achieved this suggests that whilst PKN1 and PKN2 regulate migration in a positive way, PKN3 has a role to play in inhibiting cell migration in this cell type. This points to differing roles for the PKN isoforms in the complex network of signalling pathways that contribute to breast cancer cell migration. The Boyden chamber invasion assay mimics more closely the *in vivo* mode of motility associated with cancer metastasis by looking at the ability of cells to invade into a layer of extracellular matrix proteins. Using this assay it was demonstrated that PKNs contribute to the invasion of MDAMB-468 cells. However it is not yet known which of the specific isoforms are important for invasion of these cells. In addition to the results presented here, work carried out by Dr Sylvie Lachmann in the lab has shown that PKN2 is the predominant isoform regulating migration of 5637 bladder cancer cells. This is true for both types of migration assay and additionally in these cells, substrate has less of an effect on the PKN contribution to migration than in the MDAMB-468 breast cancer cells. In

summary the nature of PKN involvement in regulating cell motility appears to be complex and the substrate and isoform specific effects observed are dependent on cell type.

Work with inhibitors of the PKNs has suggested that the catalytic activity of the PKNs is required for cell migration. In both the MDAMB-468 and the bladder cancer cells treatment with three different compounds known to inhibit PKN1, PKN2 and PKN3 significantly inhibits migration. Care needs to be taken when interpreting results obtained with chemical inhibitors since due to the highly related nature of kinases a single compound very rarely inhibits a single kinase. However this data with structurally different compounds when taken alongside the siRNA data strongly suggests that the PKNs play a role in cancer cell migration.
In an attempt to uncover why the PKNs affect cell migration the effect of PKN depletion on the cytoskeleton was investigated. A spreading assay was chosen since the ability of cells to adhere and spread is a component of the Boyden chamber migration assay that demonstrated a PKN involvement in cell migration. Whilst overexpression of PKN1 was shown in the last chapter to affect the organisation of the actin cytoskeleton in response to hyperosmolarity no evidence of an effect on the cytoskeleton or overt changes in cell morphology upon PKN depletion were observed here. However the effect of depletion of the PKNs on the cytoskeleton was only examined in spreading cells, it is possible that if one were to examine cytoskeletal rearrangements at the leading edge then a defect in the actin or microtubule networks would be evident. Closer examination of the effects of PKN depletion on the cytoskeleton, particularly real time analysis of cytoskeleton rearrangements in migrating cells would be of great interest but were unfortunately beyond the time constraints of this work.

Dissecting the precise motility signalling pathways that the different PKN isoforms lie on will be a demanding task but will undoubtedly lead to an increased understanding of the substrate and motility specific requirements demonstrated in this chapter. Following on from this work the next chapter will explore a reported target of PKN and whether this interaction contributes to the PKN functions presented so fa

Chapter 5

PKN and PLD

5 Introduction

Phospholipid metabolising enzymes such as PI3-kinase, phospholipase C and phospholipase D (PLD) generate lipid second messengers that play a crucial role in signal transduction (see 1.5.2). PLD activity produces PtdOH that can be further metabolised to produce DAG and lysophosphatidic acid (LPA), both of which also have second messenger functions. There are two mammalian PLD genes (PLD1 and PLD2) that both have splice variants, the precise roles of these splice variants is unclear. The isoforms are widely expressed but appear to have different subcellular distributions and regulation (for review see (Foster and Xu, 2003)).

Although PLD isoforms have been associated with many aspects of cell physiology of particular interest to this thesis are reports of PLD involvement in cell migration. For example in MDAMB-231 cells PLD was shown to stimulate cell migration and invasion in response to serum withdrawal (Zheng et al., 2006). This contribution to cell migration is possibly linked to the regulation of PLD by Rho and Arf GTPases (Powner and Wakelam, 2002) and their effects on the actin cytoskeleton.

PKN1 and PKN3 have been previously shown to interact with PLD1 and to stimulate PLD1 activity in vitro (Oishi et al., 2001). Additionally PKN1 was shown to mediate the α_{1A}-adrenergic receptor stimulated activation of PLD1 (Parmentier et al., 2002). The results from inhibitor and siRNA studies in the previous chapter have indicated a requirement for protein kinase N (PKN) isoforms in the migration and invasion of MDAMB-468 cells.
This chapter therefore investigates if the PKNs contribute to the migration of MDAMB-468 cells through activation of PLD.

5.1 PKN1 and PLD colocalise.

PKN1 and PLD1 have been reported to interact (Oishi et al., 2001) and it was decided to examine the cellular distribution of PKN1 and PLD isoforms to investigate if they localise to the same areas of the cell. DsRed-PKN1 was co-transfected with either GFP-PLD1a, GFP-PLD1b or GFP-PLD2 in MDAMB-468 cells. Previously it had been observed that DsRed-PKN1 translocated to punctate structures in response to hyperosmotic stress (see Chapter 3). Transfected cells were therefore either untreated or subjected to 30 minutes of hyperosmolarity prior to fixing the cells and examining slides by confocal microscopy. A degree of colocalisation was observed in most cells expressing both DsRedPKN1 and a GFP tagged PLD isoform both under basal conditions and after hyperosmotic stress. Under basal conditions both PKN1 and all the PLD isoforms had a diffuse cytoplasmic and nuclear distribution with some increased intensity of signal at points of membrane protrusion. An example of this type of localisation is seen with GFP-PLD1a and GFP-PLD2 in the top two panels of Figure 5:1. In response to hyperosmotic stress GFP-PLD isoforms were seen in some cases to translocate to punctate structures in a similar manner to PKN1. Like PKN1 expressing cells the translocation to punctate structures did not occur in 100% of cells and appeared to occur more frequently in GFP-PLD1 expressing cells. When GFP-PLD isoforms were expressed with DsRed-PKN1 it was seen that the two proteins did colocalise in these punctate structures as demonstrated by GFP-PLD1a in the bottom two panels of Figure 5:1. These observations suggest that PLD isoforms and PKN1 are closely associated under some circumstances suggesting that they may participate in the same signalling pathway in response to certain stimuli. Since results from chapter 4 suggested that PKN isoforms may play a functional role in motility of MDAMB-468 cells the localisation of the proteins at points of membrane protrusion could be relevant to a role in controlling cell motility. Following on from these colocalisation observations the importance of PLD activity for migration of MDAMB-468 cells was investigated.

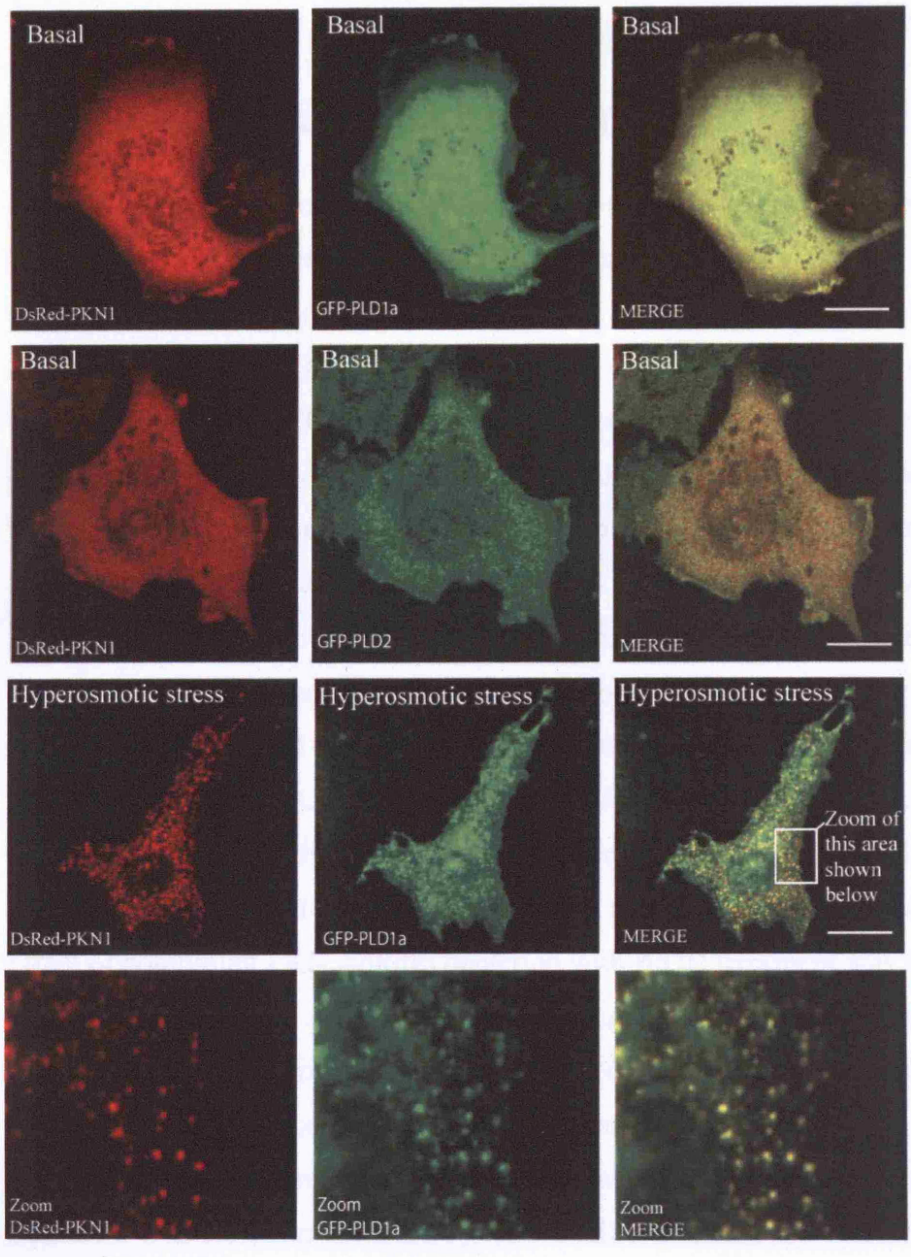


Figure 5:1 DsRed-PKN1 and GFP-PLD isoforms have overlapping localisation.

MDAMB-468 cells were transiently co-transfected with DsRed-PKN1 and either GFP-PLD1a, GFP-PLD1b or GFP-PLD2 and either untreated (basal) or subjected to 30 minutes of hyperosmolarity by treatment with 0.4M sucrose (hyperosmotic stress). All images are a representative single 1.0μM 'Z' section, the scale bar is equivalent to 10μM.

5.2 PLD mediated PtdOH production contributes to MDAMB-468 cell migration.

When cells are incubated in the presence of a primary alcohol such as butanol or ethanol these are used in preference to water in the hydrolysis reaction of PtdCho catalysed by the PLD enzyme. This results in the generation of an inert phosphatidylalcohol instead of phosphatidic acid. This phenomenon can be utilised in the "alcohol trap" assay to assess the contribution PLD mediated PtdOH production has on cell behaviour. To investigate if PLD activity is involved in the migration of MDAMB-468 cells, cells were pre-incubated with either 1% ethanol or 0.1% butanol for 1 hour prior to the initiation of a scratch wound assay that was also performed in the presence of the primary alcohols. As in the previous chapter the speed at which control cells and treated cells migrated into the wound were compared (Figure 5:2). Both EtOH and BtOH decreased the speed at which cells migrated into the wound in the otherwise confluent monolayer of cells. The presence of 1% EtOH or 0.1% BtOH resulted in a 40% and 30% decrease in speed of migration respectively. In this case the control cells were not treated with any alcohol so the experiment was repeated using Bt-2-OH as a control for BtOH. Additionally the effect of combining PKN isoform depletion with BtOH treatment was investigated. As can be seen in Figure 5:3 consistent with findings in the previous chapter, depletion of PKN isoforms results in a reduction in the speed of MDMB-468 cell migration compared to the LUC control (in this experiment 65%). The presence of BtOH results in a 53% decrease in migration speed compared to LUC transfected cells that were treated with Bt-2-OH. However there is no additive effect of both PKN isoform depletion and treatment with BtOH since the decrease in speed of migration is similar for PKN depleted cells whether they were treated with Bt-2-OH or BtOH. These results suggest that PLD generated PtdOH plays a role in controlling migration of this transformed breast cell line. Since BtOH treatment did not increase the negative effect on migration observed by PKN depletion alone it is possible that PKN and PLD are operating in the same signalling pathway to control migration. However it is also possible that the PKNs and PLD are operating in parallel pathways and no additive effect is seen due to inadequate sensitivity of the assay or one pathway overriding the effects of the other.

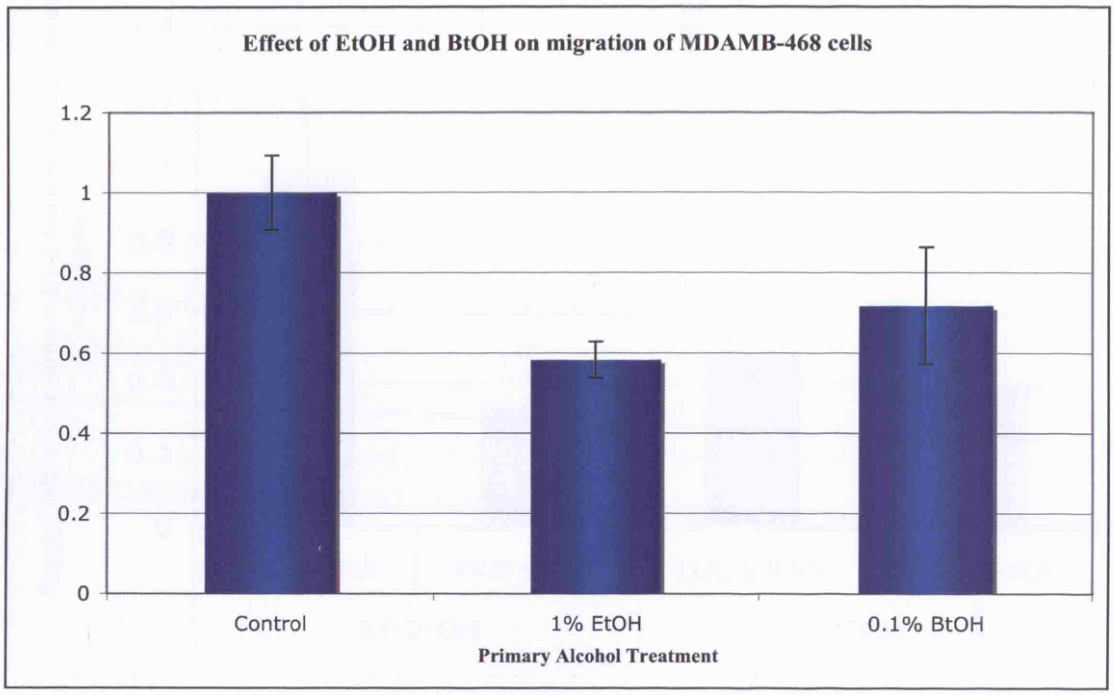


Figure 5:2 Effect of EtOH and BtOH on MDAMB-468 migration.

Confluent layers of MDAMB-468 cells were pre-treated for 1 hr as indicated prior to scratch wound assays being performed. The speed of migration is shown relative to an untreated control; n=1 performed in triplicate, error bars display the standard error.

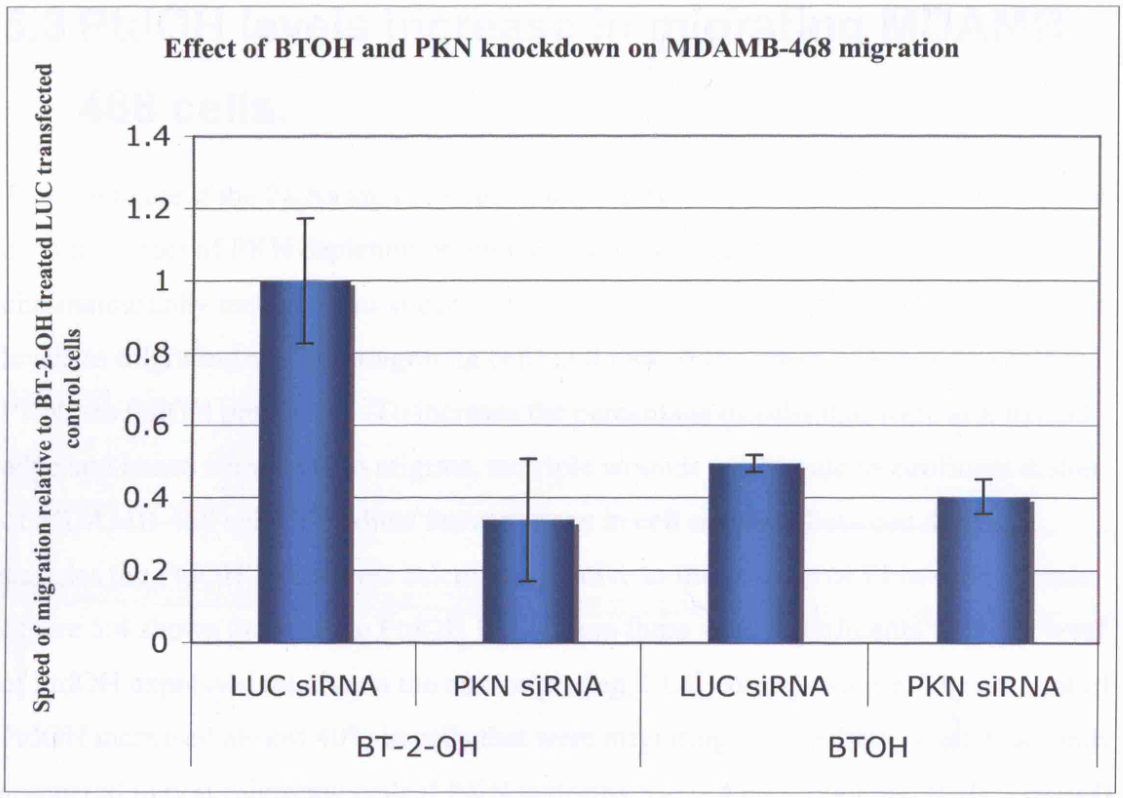


Figure 5:3 Comparing the effects of inhibition of PtdOH production and knockdown of PKN isoforms on migration.

MDAMB-468 cells were transfected with siRNA oligos, either a LUC control oligo or 3 separate oligos each targeting a PKN isoform (PKN siRNA). 72 hrs post-transfection cells were incubated in either 0.1% Bt-2-OH or 0.1% BtOH for 1 hr prior to a scratch wound assay being performed in the presence of the same concentrations of primary or secondary alcohols. The speed of migration is expressed relative to the LUC transfected/ BT-2-OH treated control cells; n=1 performed in triplicate; error bars display the standard error.

5.3 PtdOH levels increase in migrating MDAMB-468 cells.

To investigate if the PKNs are involved in the control of PLD activity in MDAMB-468 cells the effect of PKN depletion on PtdOH levels was examined. Liquid chromatography tandem mass spectrometry (LC-MS/MS) was used to measure PtdOH levels in migrating and non-migrating cells and look at the effect of knockdown of the PKNs on PtdOH production. To increase the percentage of cells that were at a wound edge and hence stimulated to migrate, multiple wounds were made in confluent dishes of MDAMB-468 cells. To adjust for variations in cell numbers between different samples the PtdOH levels were calculated relative to the amount of PI in each sample. Figure 5:4 shows the average PtdOH levels from three such experiments with the level of PtdOH expressed relative to the non-migrating, LUC control sample. The amount of PtdOH increased almost 40% in cells that were migrating into multiple scratch wounds compared to non-migrating cells if PKN isoforms had not been depleted (LUC control). Depleting the levels of PKN isoforms did not have an effect on the amount of PtdOH extracted from a confluent monolayer of cells. However depletion of the PKNs prevented the increase of PtdOH levels seen when control cells were stimulated to migrate into wounds in the monolayer. This suggests that the PKN isoforms do not affect the basal activity of PLD enzymes but that one or more of the PKN isoforms stimulates PLD activity in migrating cells.

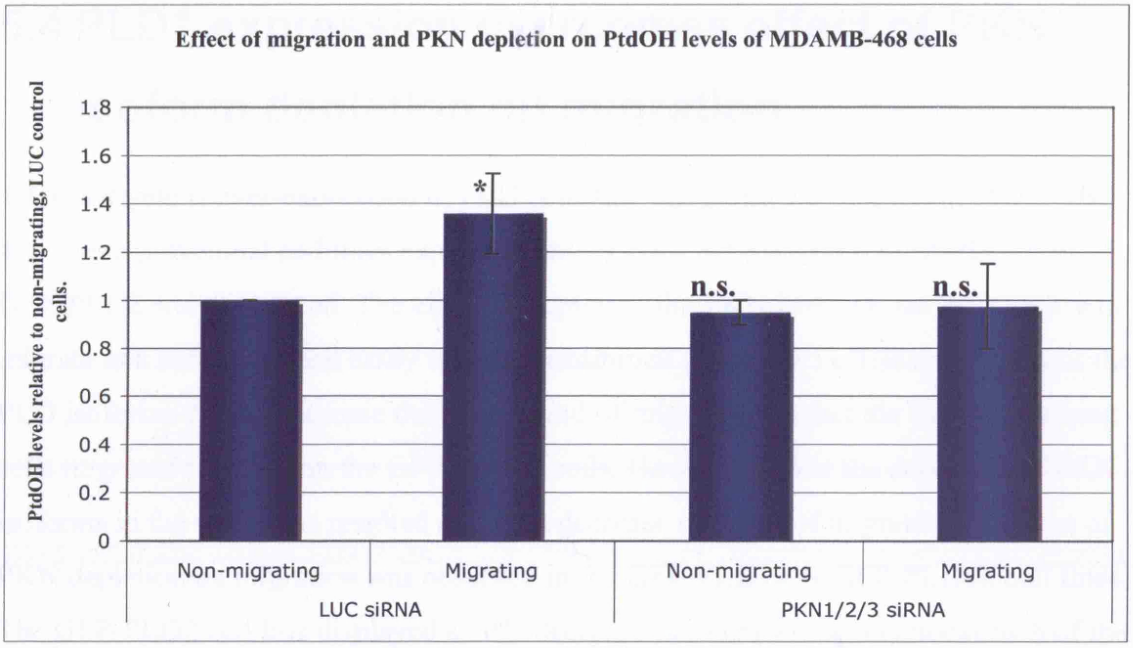


Figure 5:4 PtdOH levels in migrating MDAMB-468 cells and effects of PKN depletion.

Lipids were extracted from cell samples and analysed by LC-MS/MS. The results shown are the average PtdOH levels relative to PI levels and are expressed relative to the non-migrating, LUC control cells. Bonferroni corrected Z tests were performed; n=3; n.s. represents no significant change in PtdOH levels relative to the control; * represents $p<0.05$.

5.4 PLD1 expression overcomes effect of PKN isoform depietion on migration.

To investigate if over-expression of PLD isoforms can stimulate migration, MDAMB-468 stable polyclonal cell lines expressing either GFP, GFP-PLD1a, GFP-PLD1b or GFP-PLD2 were produced. The effect of depleting the PKN isoforms on their ability to migrate in a scratch wound assay was then examined (Figure 5:5). The expression of the PLD isoforms did not increase the basal speed of migration, in fact the PLD expressing cells migrated slower than the GFP control cells. However whilst the depletion of PKN isoforms in the GFP cells resulted in a 20% decrease in speed of migration no effect of PKN depletion on migration was observed in the GFP-PLD1a or GFP-PLD1b cell lines. The GFP-PLD2 cell line displayed a 10% decrease in migration upon knockdown of the PKNs. These results suggest that expression of PLD isoforms and in particular PLD1 can rescue the effects of PKN depletion on migration of MDAMB-468 cells. However these results are only preliminary data and this needs to be repeated so that statistical analysis can be performed and the depletion of the PKN isoforms confirmed by western blot before any firm conclusions can be made. Additionally the consequence of expressing a plasmid stably appears to affect the effect of PKN knockdown on migration since only a 20% decrease in speed of migration is seen upon PKN depletion in the GFP control cell line, this could be due to a less efficient knockdown being achieved. To try and circumvent this problem a stable inducible system of knockdown using plasmids encoding shRNA could be utilised.

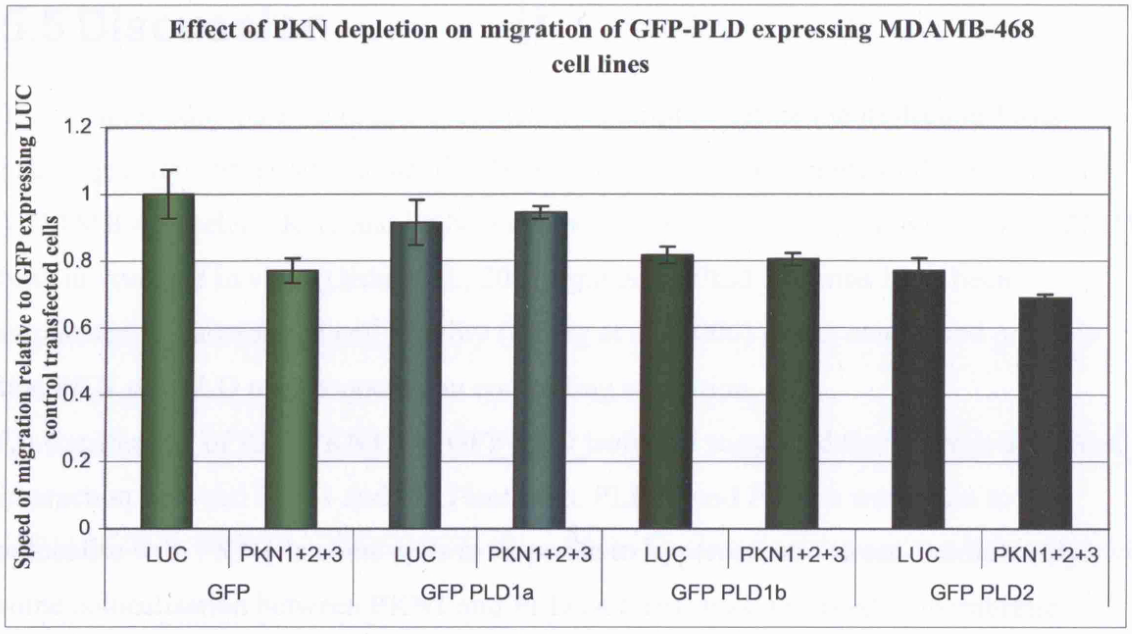


Figure 5:5 Effect of PLD expression on migration of MDAMB-468 cells.

MDAMB-468 stable polyclonal cell lines expressing GFP, GFP-PLD1a, GFP-PLD1b or GFP-PLD2 were produced. These cell lines were transfected with siRNA oligos (either LUC control or a combination of three siRNA oligos targeted against the three PKN isoforms) and a scratch wound assay was performed. The graph shows the speed at which these cell lines closed the wound relative to the GFP cell line transfected with the control siRNA; n=1 performed in triplicate; error bars display the standard error.

5.5 Discussion

This chapter adopted a candidate approach to attempt to define the molecular basis behind previous observations that the PKN isoforms may contribute to the motility of MDAMB-468 cells. PKN1 and PKN3 have been previously found to interact with PLD1 both in vivo and in vitro (Oishi et al., 2001) and since PLD isoforms have been implicated in transformed cell motility (Zheng et al., 2006) it was considered possible that PKN and PLD may cooperate in controlling migration.

Co-transfection of RFP-PKN1 and GFP-PLD isoforms suggested that there is a level of interaction between PKN1 and PLD isoforms. PLD1a and PLD1b were seen to colocalise with PKN1 in some cells in response to hyperosmotic stress. Additionally some colocalisation between PKN1 and PLD isoforms was observed in membrane protrusions in MDAMB-468 cells under basal conditions. Difficulties of co-transfecting two constructs meant that these localisation studies were not as extensive as they could have been. It would be most interesting to examine the localisation of endogenous PKN1 and PLD isoforms unfortunately though the antibodies available are of insufficient quality for use in immunoflouresence.

Directly measuring the levels of PtdOH in cells using LC-MS/MS demonstrated that PtdOH levels increase in actively migrating MDAMB-468 cells. This finding is consistent with the observation that treating cells with primary alcohols decreased the speed at which they are able to migrate. Although primary alcohols are likely to affect cellular activities other than just PLD catalysed hydrolysis of PtdCho their effect on migration when considered with the LC-MS/MS results strongly imply that PtdOH production contributes to the ability of MDAMB-468 cells to migrate. These results however do not inform us whether PtdOH acts directly to promote migration or whether it is an intermediate signalling molecule and derivatives such as DAG are the direct effectors regulating the activity and localisation of proteins such as the cPKCs.

Interestingly PKN depletion inhibited PtdOH production in migrating cells to the levels seen when cells were not actively migrating. This suggests that the increase in PtdOH production seen in migrating cells is a consequence of the presence of PKN isoforms. Since PKN1 and 3 have been shown to stimulate PLD activity it is tempting to

hypothesise that PKN activity is required for the migration stimulated increase in PtdOH production but this has not been tested here. It therefore remains possible that PKN isoforms play a scaffolding role in regulation of PLD activity.

To summarise, it appears that PLD function is required for MDAMB-468 cell migration and that PKN isoforms operate upstream of PLD in the generation of second messenger PtdOH. These results suggest that PLD lies downstream of PKN in a signalling pathway that contributes to the control of MDAMB-468 cell migration.

Chapter 6

Discussion

6 Overview

The work presented in this thesis strengthens the link between the PKNs and the cytoskeleton. The PKNs have previously been described to be activated by Rho GTPases (Amano et al., 1996b), (Flynn et al., 1998), (Watanabe et al., 1996) that are major regulators of the cytoskeleton and to interact with numerous cytoskeletal components such as the actin binding protein α-actinin (Mukai et al., 1997). The work presented here looked at the PKNs in two contexts of dynamic cytoskeleton rearrangements, hyperosmotic shock and cell motility. In the first instance overexpression of GFP-PKN1 was seen to disrupt the actin cytoskeleton in response to sucrose induced hyperosmolarity and stabilise actin filaments in response to hyperosmolarity caused by urea, highlighting the complex nature of the signalling pathways that regulate the cytoskeleton. The translocation of PKN1 to punctate structures in response to hyperosmolarity was found to be specific for sucrose over urea induced hyperosmotic stress suggesting that PKN1 is responding to mechanical stress rather than hyperosmolarity per se. The translocation observed here for PKN1 was shown to be mediated through a novel 49 amino acid region located within the PKN1 kinase domain.

In searching for a role for the PKNs in cell motility it was found that the contribution of the PKNs is cell type dependent and that the relative contribution of the three isoforms varies depending on the specific nature of cell motility being assessed. Attempts to define a molecular basis for the PKN role in cell migration pointed towards a role for the PKNs in activation of PLD isoforms in migrating cells and interestingly PLD isoforms have been implicated previously in control of the cytoskeleton (Komati et al., 2005). Further work is needed to fully understand the exact nature of PKN effects on cell motility and whether they contribute to regulation of the cytoskeleton in this context or whether they contribute to regulation of cell motility in another manner.

6.1 PKN1 and the cytoskeleton

In searching to further our understanding of previous characterisation of PKN1 as a stress responsive kinase it was found that over-expression of GFP-PKN1 altered the rearrangements of the actin cytoskeleton seen in response to hyperosmolarity. Given the PKNs control by Rho GTPases it is perhaps not surprising that overexpression would affect the cytoskeleton since the presence of higher concentrations of specific effectors of the Rho GTPases is likely to disrupt the fine balance that exists normally to control the cytoskeleton.

Depletion of PKN isoforms by siRNA resulted in a decrease in the ability of MDAMB-468 cells to both migrate and invade. It would be interesting to examine further the dynamics of cytoskeletal regulation in these cells. Recent advances in optical imaging means that the visualisation of metastasising cells is now a possibility (Sahai, 2007). Combining this with the expression of fluorescent tagged proteins would allow effects of PKN depletion on the cytoskeleton of invading cells to be examined. Although no overt affect of PKN depletion on the actin or microtubule networks was observed in spreading cells it is possible that an examination of the cytoskeleton in migrating or invading cells would highlight defects. Live imaging using tagged actin or tubulin would be a powerful way of examining cytoskeleton dynamics at the leading edge. Additionally it would be interesting to probe the link between PKNs and cell surface receptors that mediate cell-cell and cell-matrix adhesion and associated proteins that provide a link between these receptors and the cytoskeleton. Sites of cell-matrix adhesion such as focal adhesions have been suggested to allow integrins to act as mechanosensors (for review see (Katsumi et al., 2004)) and the PKN1 translocation in response to hyperosmotic stress was suggested by results presented in chapter 3 to be due to mechanical stress caused by membrane impermeant solutes such as sucrose. Perhaps this translocation of PKN1 and associated signalling events is activated downstream of particular integrin activation. This could be investigated through the use of integrin blocking antibodies (Crowe and Ohannessian, 2004) to examine effects of inhibiting specific integrin dimer activation on PKN1 translocation. Supporting the idea that the PKNs are activated downstream of specific cell surface receptors is the finding

that the PKN contribution to migration of MDAMB-468 cells is substrate dependent. The strongest effect of PKN depletion on migration was seen when cells were migrating on fibronectin perhaps implicating PKN activation downstream of fibronectin binding integrins such as α4/5β1 or αV/IIbβ3. Differential activation of integrins has been shown previously to regulate cell motility by selectively modulating the activities of Rho GTPases and their effectors. For example in squamous epithelial cells collagen α2β1 integrin binding activates RhoA and slows migration whereas laminin-5-α3β1 integrin binding activates Cdc42, Rac and their downstream effector PAK1 to stimulate migration (Zhou and Kramer, 2005).

6.2 PKN in motility

Work carried out in our lab with transformed breast and bladder cell have suggested that the PKNs can contribute to cell motility. Data from siRNA studies presented in chapter 4 suggest that the isoforms display redundancy in some contexts such as the Transwell migration assay and that the involvement of the PKNs is cell type dependent. At least for migration in a scratch wound assay the catalytic activity of PKN appears to be important although this does not rule out the PKNs also having scaffolding roles to play in the signalling pathways that control cell motility. Care does need to be taken when interpreting these results from siRNA and inhibitor studies since both oligos (Jackson and Linsley, 2004) and kinase inhibitors are known to have off-target effects. The possibility of off-target effects were minimised in these studies by using low concentrations of oligo (5nm) and ensuring that the sequences were specific for the intended target by using the NCBI blast resource. However to further strengthen these findings it will be necessary to use multiple siRNA oligos and ideally to also perform rescue experiments with siRNA resistant PKN isoforms.

Encouragingly though the PKN isoforms have been previously reported to be involved in the migration and metastasis of astrocytes and PC3 cells respectively (Bourguignon et al., 2007), (Leenders et al., 2004). Additionally phenotypes of the $PKN^{-/-}$ Drosophila and $PKN1^{-/-}$ mice further suggest a role for the the PKNs in migration. Drosophila

PKN$^{-/-}$ are embryonic lethal due to defects in dorsal closure (Lu and Settleman, 1999). Dorsal closure involves migration of the lateral epidermal flanks and dynamic changes in cell shape to close a hole in the dorsal epidermis occupied by an epithelium called the amnioserosa. The dorsal closure process has been likened to migration of epithelial cells in other organisms and in a similar manner Rho GTPases have been shown to play an important role (Wood et al., 2002). PKN1$^{-/-}$ mice do not display an overt phenotype, perhaps due to the presence of PKN2 and PKN3 that in some contexts (for example migration through a Transwell) may have redundant function with PKN1. Interestingly though these PKN1$^{-/-}$ mice do have a decrease in the number of circulating neutrophils in response to an inflammatory stimulus. This has been hypothesised to be due to defects in the migration of neutrophils out of the bone marrow and into the circulation (Dr Casamassima, personal communication).

6.3 PKN specific domains

The PKN isoforms like many proteins have distinct domains that allow their activity and localisation to be precisely regulated. These domains can therefore be expected to contribute to the functioning of the PKN isoforms in distinct signalling pathways. The work presented in chapter 3 demonstrated that a novel 49 amino acid region located within the kinase domain of PKN1 is required for the translocation of PKN1 in response to hyperosmotic stress. The highly related kinase domain of PKCζ however does not respond in a similar fashion to hyperosmolarity. To confirm the importance of this domain for control of PKN1 localisation it would be elegant to be able to alter the behaviour of the PKCζ kinase domain to resemble that of PKN1 by mutation of the relevant amino acids. PKN2 has been found previously to also translocate to punctate structures in response to hyperosmotic stress and as you would predict these 49 amino acids are highly conserved between PKN1 and PKN2. However it is clear that despite some similarities in function between the PKN isoforms they are also capable of having distinct effects. In the context of cell motility it has been found in PC3 cells that PKN3 is required for invasive growth but PKN1 and PKN2 are not (Leenders et al., 2004).

Work presented here suggests that the migration of a sheet of MDAMB-468 cells is predominantly controlled by PKN1 and PKN2. In contrast in a Transwell migration assay where cells are migrating as single cells the isoforms appeared to have entirely redundant functions. It is not clear from these experiments whether in these contexts the PKNs are operating within the same pathway or in parallel to each other. To gain a handle on this it might be informative to examine which Rho GTPases the PKN isoforms are operating downstream of in response to motility stimuli since the Rho family GTPases have distinct roles in cell motility (see 1.8.2). The requirement for PKN in the dorsal closure of Drosophila embryos was recently shown to be dependent on Rho but not Rac binding (Betson and Settleman, 2007). Additional domains of the PKN isoforms such as the proline rich domains of PKN2 and PKN3 are also likely to play a role in controlling the action of the PKNs. The proline rich regions in particular have been shown to allow binding of SH3 domain containing proteins that may aid the formation of isoform specific signalling complexes (Quilliam et al., 1996) (Braverman and Quilliam, 1999) (Shibata et al., 2001) and contribute to isoform specific functions.

6.4 Signalling downstream of PKN

Signalling pathways downstream of the PKNs remain broadly undefined although several links have been made to the MAPK cascades and in particular the p38 pathway (Takahashi et al., 2003). Interestingly the recruitment of PKN1 to a distinct compartment in response to hyperosmotic stress has been linked to signalling to the JNK cascade. Work by Dr Cassimassima in our lab has highlighted that the phosphorylation of both MKK4 and JNK in response to hyperosmolarity is reduced in PKN1$^{-/-}$ MEFs. Although this phenotype is unstable in culture, perhaps due to compensation by PKN2 and PKN3, it has been found *ex vivo* that the same deficiency in JNK phosphorylation is seen in the heart in response to ischemia/reperfusion. Further work identified the Rac dependent assembly of a complex containing JNK, JIP (JNK interacting protein) and MKK4 (Dr Casimassima, personal communication). Interestingly JNK has been suggested many times to be important in cell motility (reviewed in (Xia and Karin,

2004)) however no effect of either PKN depletion or inhibition on the MAPK cascades has been observed in migrating, transformed bladder cells (Dr Lachmann, personal communication). However defects in MAPK signalling could be localised to events at specific cellular locations such as recruitment of phosphorylated JNK to the leading edge of cells. Hence microscopy studies might be more informative than examining the phosphorylation state of components of the pathways in the whole cell population. Results from chapter 5 suggest that one of the important downstream targets of PKN in cell migration is PLD. The results presented suggest that PLD activity is required for the migration of MDAMB-468 cells although this is contradictory to findings in HeLa cells that demonstrated that PLD1 regulated cell migration in a lipase activity independent manner (Kim et al., 2006). In our system inhibition of PtdOH production by primary alcohols decreased cell migration and most interestingly PtdOH levels were seen to increase in migrating cells in a PKN dependent manner. Perhaps the link between PKN and PLD is of relevance to the phenotype observed in PKN $^{-/-}$ mice of decreased circulating neutrophils since PLD has been suggested to regulate the migration of neutrophils (Powner et al., 2007). Colocalisation of PKN1 and PLD was observed particularly at the points of membrane protrusion and in response to hyperosmotic stress. It would be most interesting to examine the localisation of endogenous proteins. It would also be interesting to perform immunoprecipitations to examine if the previously reported interaction between PKN1 and PLD increases in response to migratory stimuli. Initial experiments suggested that PKN knockdown can be rescued by expression of PLD isoforms particularly PLD1 although these can only be considered as preliminary results and further experiments are needed to confirm this observation. It would also be interesting to examine if the migration of the GFP-PLD expressing cell lines is compromised by inhibition of the PKNs. Taken together though these results implicate PKN isoforms in signalling upstream of PLD to regulate migration of these transformed breast cells. It would be interesting to examine which of the PKN isoforms contributes to this signalling pathway. One hypothesis is that it would be PKN1 and/or PKN3 since these are the isoforms previously demonstrated to interact with PLD1 (Oishi et al., 2001).

6.5 PKN and the cell cycle

Interestingly knockdown of the PKNs although not formally investigated here showed no signs of inhibiting the cell cycle since 72 hours post-transfection there were similar numbers of LUC control or PKN siRNA treated cells. This is in contrast to HeLa S3 cells where knockdown of PKN2 was seen to slow the cell cycle due to a block at the G2M transition (Schmidt et al., 2007). In vascular smooth muscle cells however the phosphorylation of Cdc25c by PKN1 results in a delay to mitosis (Su et al., 2007). It therefore appears that the PKNs have a cell type specific role in control of the cell cycle. Interestingly it has been suggested that cell cycle delay is one of the consequences of exposure to hyperosmolarity (Dmitrieva et al., 2001). This raises the intriguing possibility that PKN1 translocation to punctate structures under hyperosmotic conditions may be linked to a signalling requirement for delay of the cell cycle. Perhaps by sequestering PKN1 in a discrete compartment/complex the cell cycle is delayed allowing the cell time to adapt to the stress conditions. Alternatively the PKNs have been linked to apoptosis (Takahashi et al., 1998) (Sumioka et al., 2000) and this is also a consequence of prolonged hyperosmotic stress (Reinehr and Haussinger, 2006). The recruitment of PKN1 to a distinct compartment along with components of the JNK cascade supports the idea that PKN1 may be involved in apoptotic signalling in response to hyperosmolarity since JNK has been shown to have both pro and anti apoptotic roles (Lin, 2003).

6.6 Future directions

The relevance of the PKN1 translocation to punctate structures in response to hyperosmolarity is intriguing and as yet not understood. It would be most interesting to utilise siRNA to look at the effects of PKN1 depletion on gene expression, using microarrays, in response to hyperosmolarity. In a similar way 2-D protein gels could be used to investigate protein modifications mediated by PKN1 in response to hyperosmolarity. These techniques could be expected to further our understanding of the

role of PKN1 in the hyperosmotic stress response and may also provide possible targets of PKN1 signalling in other contexts too such as cell motility.

Pathologically cell motility is of exceptional importance since the ability of neoplastic cells to migrate out of the primary tumour mass and colonise other sites in the body is frequently the cause of patient mortality (Sporn, 1996). Despite evidence presented here suggesting that the PKNs contribute to the ability of transformed breast cells to migrate and invade *in vitro* this has yet to be tested *in vivo*. PKN3 has been shown previously to be required for the metastasis of PC3 cells transplanted into mice (Leenders et al., 2004). It would be interesting to test the contribution of the PKNs in metastasis of transformed breast and bladder cells.

The link between PKNs and signalling in response to steroid hormones would be an interesting area of investigation particularly as PKN1 has already been described to activate the androgen receptor in PC3 cells. Aberrant hormone signalling is often responsible for the proliferation of neoplastic cells, classically those cells of hormone responsive tissues such as the breast and prostate. Interestingly though the proliferation of transformed bladder cells has recently been linked to androgens and the androgen receptor (Miyamoto et al., 2007) perhaps explaining why bladder cancer is almost four times as common in men than women (Jemal et al., 2006).

To conclude it is clear that the PKNs have diverse roles to play in signal trasnduction. Elucidation of their targets and interaction partners will help further our understanding of their role in controlling physiological responses to specific stimuli. This thesis has primarily focused on their potential role in control of the cytoskeleton and has revealed isoform and cell type specific roles for the PKNs in control of cell motility. The contribution of PKN signalling to the migration of MDAMB-468 cells is suggested to be mediated at least in part through activation of PLD although the downstream targets of PLD relevant to migration in this system are as yet unidentified. Figure 6:1 proposes a model for the PKN/PLD contribution to cell migration.

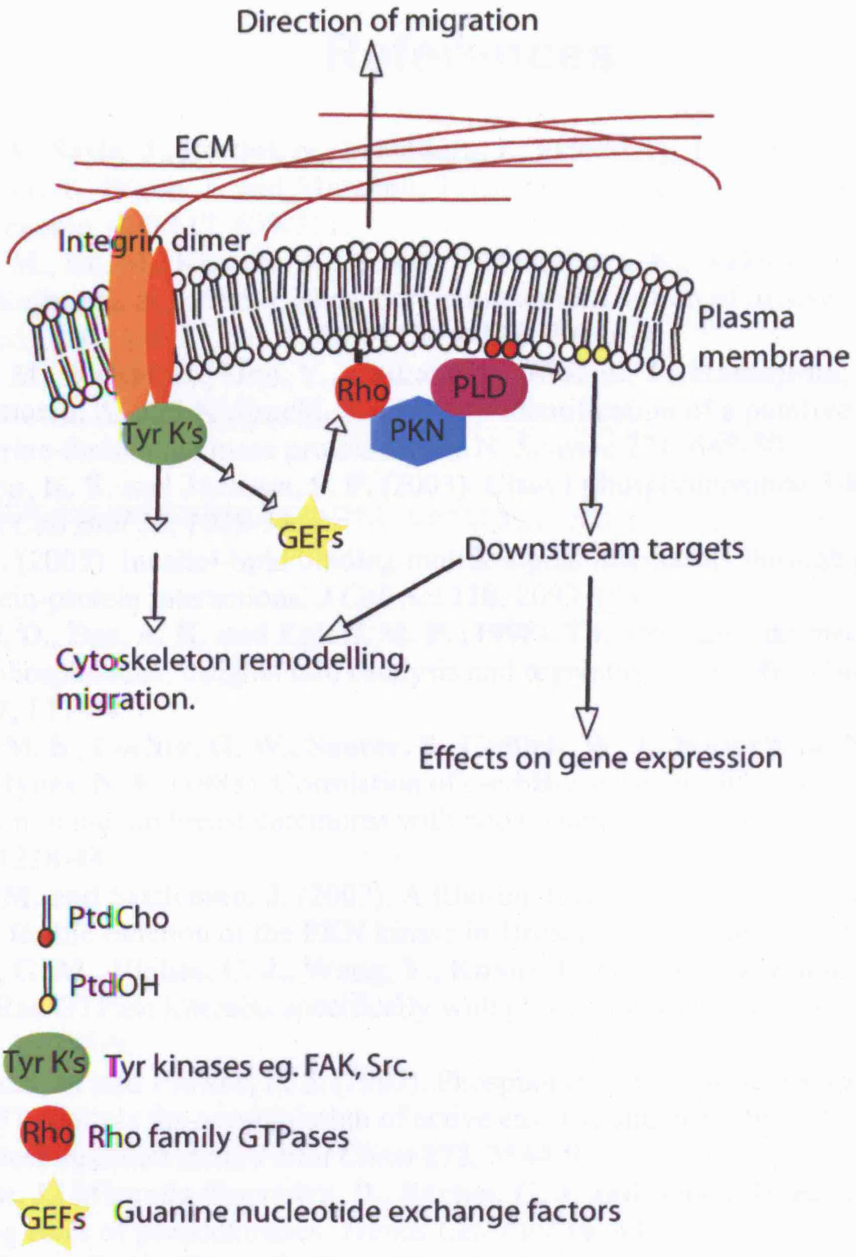


Figure 6:1 Model of PKN/PLD involvement in cell migration.

Activation of Rho family GTPases downstream of integrin engagement with ECM components is hypothesised to result in recruitment and activation of PKNs at the plasma membrane along with PLD. The resulting PtdOH produced affects the activation and localisation of multiple downstream effectors to bring about changes in the cytoskeleton and gene expression to regulate migration.

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